

Brief Report

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Brief Report

Assessment of Nitric Oxide Release *In-Vitro* via Low-Level Light Irradiation, Equivalent Blue or Red Visible Light Within Daylight

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Abstract

Conflicting evidence exists on whether blue or red light modulate nitric oxide (NO) release within skin cells. This study shows that broad-spectrum blue or red (including infra-red) light at typical low-level environmental exposures fails to significantly increase NO release in skin cell monolayers compared to unexposed controls up to 2 hours post-exposure. We discuss observed discrepancies between our work and recent studies presenting measurable upregulation, noting that significant NO induction typically requires high-powered light emitting diode (LED) or lasers used in clinical settings within a specific narrow spectral band. Thus, while our findings show no significant effect, they provide an important counterpoint for public health discussions on visible light exposure at terrestrial levels, particularly low-level exposures.

Keywords: nitric oxide; nitrite; photo biomodulation; skin; cardiovascular disease

1. Introduction

Originally regarded as an atmospheric pollutant, nitric oxide (NO) has undergone reappraisal and is now recognised as a potent biological signalling molecule [1]. Alongside local effects in wound healing, melanogenesis and lipogenesis [2–4] NO facilitates extracellular cross talk modulating blood flow within arteries [5], with loss implicated in hypertension [6,7]. This is mediated by endothelial secretion of NO, which diffuses to adjacent vascular smooth muscle cells (VSMCs) eliciting vasodilatory response [8]. The activity of nitric oxide synthases (NOS), enzymes responsible for NO production within the endothelium, play a critical role in this process. However, NOS activity declines with age, which can impair vascular function [9,10].

Liberation of NO from salts held at high concentration within keratinocytes (the primary skin cell making up 90% of the skins epidermis) by UV provides secondary effect on VSMC [11,12] and may be beneficial with highest impact within subsets of the population where enzyme-based response has waned [9,10]. Recent studies have suggested that light emitting diode (LED) and lasers emitting longer wavelengths, specifically blue (400–500nm), red visible light (620–780nm) and near infra-red (780–1000nm) may also facilitate NO release via non-enzymatic mediators [13,14]. Should such a response be recapitulated via exposure of skin to visible light as part of low-level solar exposures (for example morning or evening where significantly lower ultraviolet B irradiance occurs outside the midday peak) [15,16]. This represents an important consideration for public health, as ultraviolet radiation exerts a range of deleterious effects on skin cells under high or prolonged exposure, including the modulation of inflammatory pathways, induction of photoimmunosuppression, and acceleration of photoaging [17]. Moreover, ultraviolet radiation is recognised as a 'complete carcinogen' capable of both tumour initiation and promotion with

numerous epidemiological studies demonstrating strong associations with increased incidence of basal cell carcinoma, squamous cell carcinoma, and malignant melanoma [18].

In this study, we assess NO release *in vitro* by visible blue or red/infra-red light derived from a filtered solar simulator light source which mimics terrestrial sunlight levels. Here we opt for these exposures on the basis that previous work suggests NO is induced with negligible genotoxic effect [9,19]. We report no detectable increase in NO up to 2 hours after exposure.

2. Materials and Methods

2.1. Primary Cell Isolation and Culture

Primary keratinocyte, fibroblast and endothelial cell lines were isolated from two white skinned (Fitzpatrick scale type I and II) non-related neonatal foreskins (≤ 1 month old) collected post-circumcision and grown in culture, as previously described [9,19]. Informed consent was obtained prior to tissue collection, under approval from the South-Central Berkshire B Ethics Committee (22/SC/0411, IRAS ID 31832). For experiments, cells were seeded at a density of 250,000 cells per well and cultured for four days to reach confluence.

2.2. Exposure of Cell Cultures to Light

Visible light exposures were performed following removal of cell culture media and replacement with PBS +/- (phosphate-buffered saline with calcium and magnesium) (CSR156 Appleton woods™) on cell monolayers, as preliminary work and other studies have shown light exposure can induce the photodegradation of media constituents such as tryptophan, resulting in the liberation of hydrogen peroxide (H_2O_2) and subsequent non-physiological oxidative stress in cell monolayers in turn also upregulating reactive nitrogen species (RNS) and NO [20,21]. Exposures were conducted using a SOL-2 solar simulator (Dr Höne AG UV-Technologies™), modified to include an internal fan to dissipate heat. Desired spectral regions were isolated using a combination of long-wave and band-pass filters (Figure 1). Filters were mounted on shielded lids providing a gap between plate and filter for air flow to prevent excess heat, previously identified as arising from radiation absorbed by the filter when positioned too close to the cells [19]. Cell monolayers were exposed to either 4.86 J/cm^2 blue light (400-510 nm) at an irradiance of 5.40 mW/cm^2 , or 20.32 J/cm^2 10.14 J/cm^2 red (600-780 nm), and 10.18 J/cm^2 infra-red (780-900 nm) at an irradiance of 33.9 mW/cm^2 . Positive controls, known to induce NO release through increased fluorescence from probes DAF-FM and DAX-J2 used to detect presence [19] were incorporated into the study, via use of the BIOSUN UV device (VILBER LOURMAT™). The positive controls were irradiated with 1 J/cm^2 UV-B. The BIOSUN incorporates live dose and temperature monitoring ensuring consistent dose delivery, thus accurate delivery of positive controls was ensured.

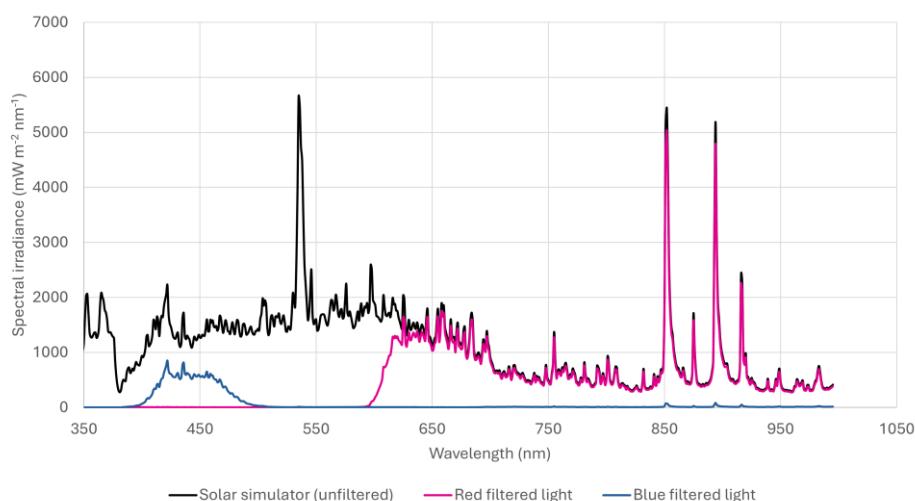


Figure 1. Schematic representation of the modified SOL-2 solar simulator (Dr Honle AG UV-Technologies) spectral output used to deliver daylight-equivalent visible light. Long-pass and band-pass optical filters were applied to isolate blue (400-510 nm), red (600-800 nm), and near-infrared (800-900 nm) spectral regions, indicated by the 'blue-filtered light' and 'red-filtered light' bands above. The filtered light set-up incorporated continuous airflow to prevent thermal artifacts, while placement of filters to allow air flow minimized heat transfer and ensured homogenous irradiance across wells.

2.3. Nitric Oxide Detection

NO levels were assessed using DAF-FM diacetate (DAF-FM DA; Molecular Probes, D23844) and DAX-J2 Red (16301-AATB, AAT BIOQUEST™), with either propidium iodide (P3566, THERMO fisher™) or CELL-TOX green (G8741, PROMOCELL™) added respectively prior to flow cytometry but after visible light exposure to remove dead or dying cells. Use of alternate probes exciting within the blue and red spectrum respectively after light exposure, allowed quenching of the probe (providing erroneous results) to be subverted when blue and red light were administered, as we experienced within previous work [19].

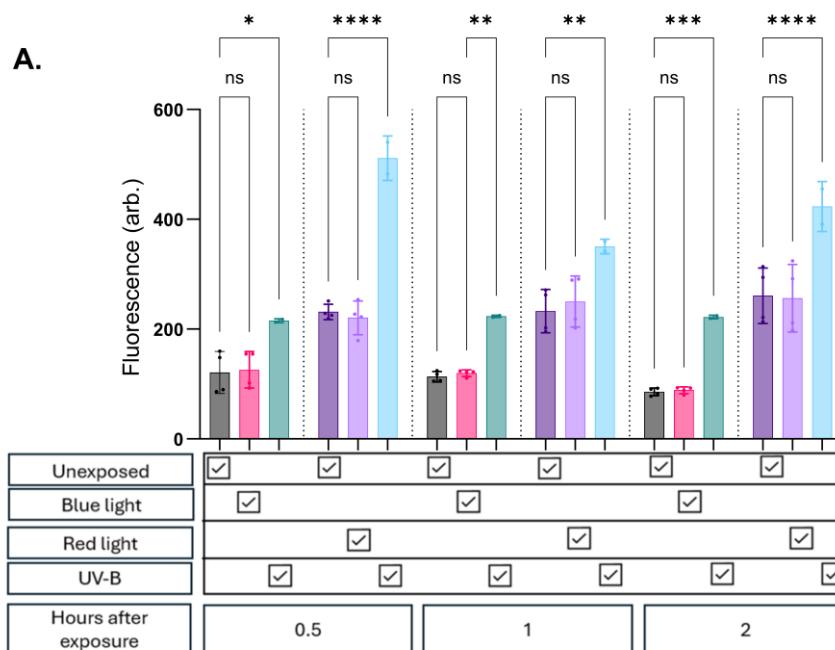
2.4. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism™ using an ordinary one-way ANOVA, with mixed effects model. Significance was set as $p < 0.05$.

3. Results

At all evaluated timepoints in all cell lines, there was no significant change in NO expression whether exposed to blue or red/infrared light compared to unexposed controls (Figure 2a, b and c). Potential thermal effects which could influence the results were accounted for, minimising confounding due to heat exposure.

As expected, exposure to UV-B consistently induced a significant increase in fluorescence in both keratinocyte and endothelial cell lines. In contrast, fibroblasts exhibited a lower propensity for NO upregulation following UV-B exposure. These cells showed modest trends up to 30 minutes post-exposure, with variable (significant and non-significant) increases observed at later timepoints (Figure 2c). Importantly, foetal bovine serum (FBS) and phenol red were removed from the media prior to probe assessment to prevent interference.



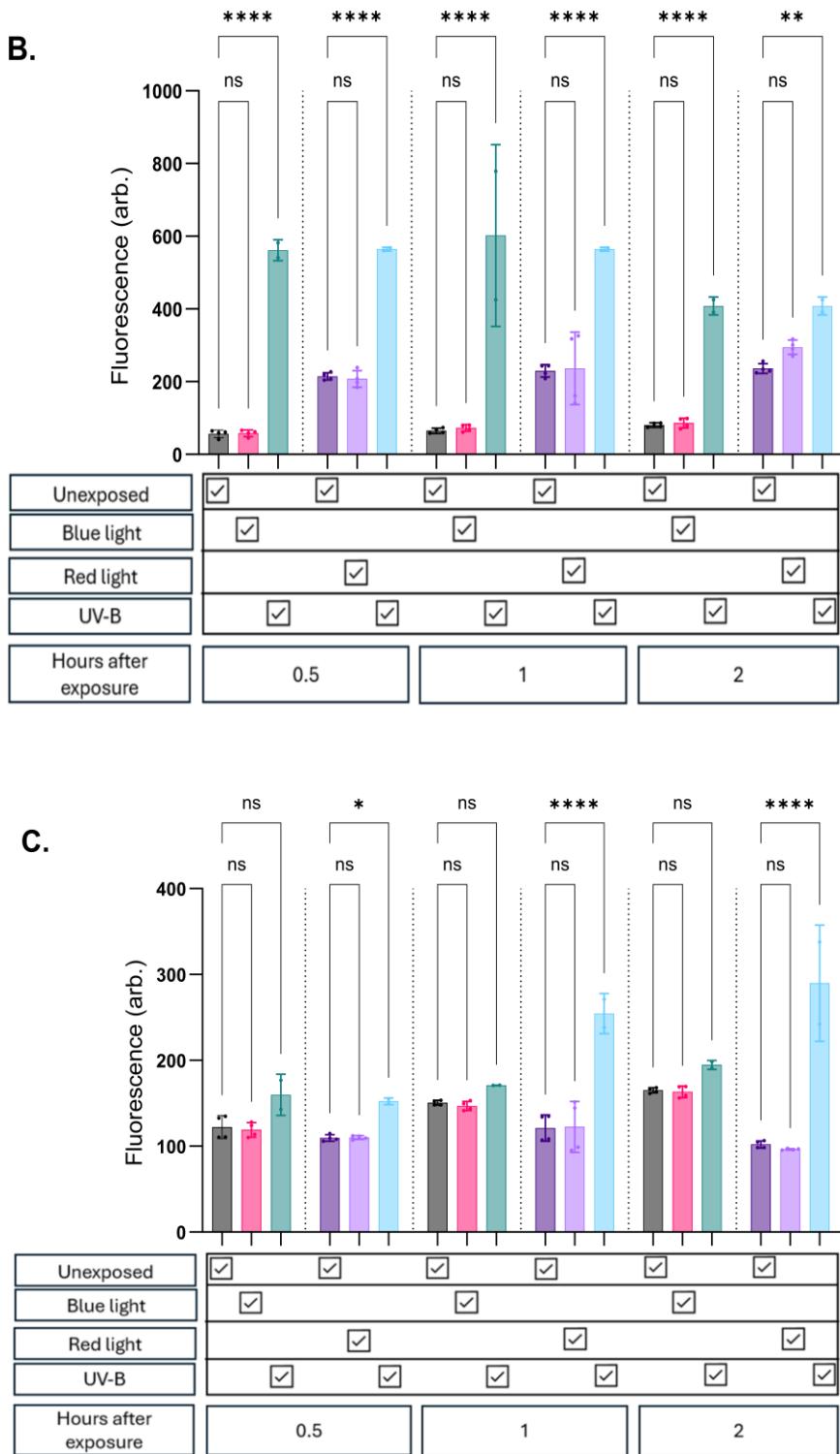


Figure 2. (A–C) Fluorescent probes DAX-J2 Red and DAF-FM diacetate used to detect NO generation in cells exposed to blue (400–500 nm) or red/infrared (>600 nm) light, respectively. DAX-J2 fluorescence were recorded at peak emission of 609 nm, DAF-FM fluorescence at 515 nm. NO levels were measured for up to 2 hours post-exposure in neonatal keratinocytes (A), endothelial cells (B), and fibroblasts (C). Across all cell types, visible light did not elicit a significant increase in NO, indicating that low-level, full-spectrum sunlight-representative blue or red light does not modulate NO bioavailability. UV-B (1 J/cm²) served as a positive control, statistical analysis was performed in GraphPad PrismTM using an ordinary one-way ANOVA with a mixed-effects model; significance was defined as $p < 0.05$.

4. Discussion

Our findings diverge from previous studies that report significant NO release following exposure of cells to blue or red light. In non skin-based cell types, Zhang et al demonstrated this at 670 nm observing NO induction following 7.5 J/cm^2 exposure, while Rohringer et al found NO induction at 24 J/cm^2 of 516 nm or 635 nm light [22,23]. Considering this it is feasible that skin cells (routinely exposed to visible light *in vivo*) may be more resistant to light-induced NO release than other cell types.

This interpretation is challenged by recent work from Barolet et al and Albers et al who demonstrate significant NO production in human skin cells following exposure to visible light [24,25]. Albers et al suggests effect in keratinocytes at 453 nm after 100 J/cm^2 exposure [24], while Barolet uses a multi-wavelength LED array (455, 650, and 850 nm), delivering blue and red light at an irradiance of 20 mW/cm^2 to achieve a dose of 15 J/cm^2 over 12.5 minutes. Barolets study is particularly interesting given the experimental set up closely matches our own in terms of NO detection methodology (via use of DAF-FM diacetate) and incorporation of cell lines in part derived from donors of identical anatomical origin, age, and gender. Additionally, the study not only accounts for unwanted effects from thermal and cytotoxic insults (that may occur as part of light exposure regimens) through a 'water-cooled irradiation system' and viability assays but goes one step further, validating the response from the probe through NO induction with the NO scavenger Carboxy-PTIO potassium salt (CPTIO). Here the researchers rule out other signalling intermediates such as ROS which is suggested to also interact with NO probes at a lower threshold [25].

It is likely that delivery of visible light outside these specific narrow bands over much broader spectral ranges at levels representative for low level terrestrial sunlight exposure goes some way to explaining the lack of response seen in the results presented here. Proof of this lies in Barolets study where blue light exposures were ~ 3 times higher dose applied when compared against our own work (4.86 J/cm^2 vs 15 J/cm^2), this may be sufficient to initiate photolysis of S-nitrosothiols (RSNOs) [13,26,27]. Inferred as RSNO photolysis is tied into blue light release of NO, owing to the fact that the metabolite requires lower energy than photoreduction of nitrite (23–34 kcal/mol) [28–30]. Further supporting this interpretation is prior cited work in cutaneous and non-cutaneous cell types, including that by Albers et al who demonstrate NO release at $453 \pm 10 \text{ nm}$, at doses upwards of 20 J/cm^2 [24]. Together, these findings suggest low level exposures to sunlight may not be sufficient to elicit biologically meaningful NO production without UV (applied sun protection, diurnal or seasonal drop), raising important questions about the physiological plausibility of this mechanism under certain real-world conditions.

An additional consideration for future work is the addition of trace biological cofactors absent in the current model but present in whole tissue and blood. This is inferred given reports suggesting interplay with protein and non-protein bound intermediates lost as part of the cell isolation process [26,27,31]. Opländer et al highlights this for visible light exposures with the addition of copper (Cu) catalysing RSNO decomposition to NO under blue-light exposure [27]. This is feasible *in vivo*, as both free copper and copper-containing enzymes may contribute to nitric oxide-related chemistry, although they represent distinct entities. Free copper ions are present within the epidermis at low micromolar concentrations (approximately $1\text{--}30 \mu\text{M}$), whereas copper-containing enzymes such as ceruloplasmin circulate in plasma at slightly higher levels ($12\text{--}25 \mu\text{M}$) [27]. Similarly, Dejam et al demonstrate thiols lost during purification of cell isolation and culture markedly enhance photolytic nitrate breakdown, increasing NO generation and RSNO formation [31]. Although these studies provide an interesting counterpoint to our own work and provide mechanistic proof-of-principle, as experimental levels are supraphysiological (and likely enhance efficiency), it remains important to follow up claims under conditions approximating environmental levels found *in vivo*. Case in point is Opländer's work that utilises $100 \mu\text{M}$ copper chloride (CuCl_2), greater than physiological levels highlighted earlier [27].

Red and near-infrared light doses used in our study were broadly comparable to those reported by Barolet [25] but applied over broader spectral regions rather than specific narrow wavebands.

Here, higher dose thresholds for wavelengths understood to interact with mitochondrial chromophores may be a factor [32,33]. These interactions may therefore lead to the photodissociation of NO that is transiently bound to cytochrome c oxidase, a key enzyme of the mitochondrial respiratory chain, [34]. Thus, although we did not observe significant NO production under red light exposure, broad spectrum wavebands opted for over narrow wavebands may be a factor. Additionally, as mitochondrial NO may account for a small proportion of total NO production in some cells such as endothelial cells which possess only 2–5% the mitochondrial content of other cell types relying primarily on glycolysis for ATP production [35], this may also be a factor explaining the results in this study.

Finally, a critical but often overlooked factor in *in-vitro* studies of light-induced NO release is the medium used during irradiation. Several studies, including those by Albers, Rohringer, Zhang, and Oppländer, clearly state that cells were exposed in phosphate-buffered saline (PBS) [22], [23,24,27,36]. However, many studies do not specify whether the culture medium was removed or replaced prior to light exposure which is a significant omission, as media components such as tryptophan can degrade under light to generate ROS, RNS, or NO indirectly [37]. Without standardisation in exposure conditions, it becomes difficult to confidently attribute observed signals to direct NO production. We found that chronic exposure of media, to blue light in particular, prior to addition of cells led to complete cytotoxicity (data not shown), thus even transient exposures from LED's may have considerable effects on inflammatory pathways mediating ROS, RNS and NO induction.

5. Conclusions

The concept of visible-light-induced nitric oxide release remains of practical importance. While several studies have demonstrated NO generation using LED-based light sources, these findings have not been consistently replicated under conditions that reflect low-level environmental exposures. Such low-level exposures are relevant to mimic natural sunlight exposure while avoiding peak UV exposure. Discrepancies in exposure conditions including light source, dose, spectral power distribution and cell media may underlie conflicting reported results.

Future work should aim to replicate these effects in more complex models that incorporate key biological cofactors, maintain native tissue architecture, and simulate environmental light conditions. This will be essential for an assessment of the potential for visible light to elicit meaningful NO-mediated effects outside of controlled clinical settings

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Institutional Review Board Statement: All methods described above were performed in accordance with relevant guidelines and regulations. For human tissue research ethics approval was granted under South-central Berkshire B ethics committee 22/SC/0411, IRAS ID 31832. Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request. All methods described above were performed in accordance with relevant guidelines and regulations.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NO	Nitric oxide
LED	Light Emitting Diode
NOS	Nitric oxide synthase
UV	Ultraviolet
H_2O_2	Hydrogen peroxide
RNS	Reactive nitrogen species
CPTIO	Carboy PTIO potassium salt
ROS	Reactive oxygen species
RSNO	S-nitrosothiols
Cu	Copper
$CuCl_2$	Copper Chloride
FBS	Fetal Bovine Serum

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