Low-dose daylight exposure induces nitric oxide release and maintains cell viability in vitro

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Abstract

Any potential positive effects conferred via sunlight exposure have to be carefully balanced against carcinogenic effects. Here we provide evidence UK sunlight exposure upregulates the cardio protectant nitric oxide (NO) within *in vivo* skin cell lines with negligible increases in DNA damage and cell death at 1 SED, when compared against unexposed samples. The whole of the ultraviolet A (UV-A) spectrum appears to be responsible for NO release, with other wavelengths outside the UV spectrum mitigating adverse effects derived through ultraviolet B (UV-B). These results support further *in vivo* work, which could be of benefit for demographics such as the elderly (that exhibit a natural decline in NO bioavailability).

Introduction

Over-exposure to sunlight increases risk of skin cancers, erythema, cataracts, premature skin aging and a weakened immune system [Kricker et al 1994, Rittie & Fisher 2015, Zigman 1983]. However, human life evolved under the sun and insufficient exposure also harbours adverse health consequences. Vitamin D circulation promoting healthy bones and prevention of rickets, alongside the blood pressure lowering effect of UV-A are, perhaps, the most well documented examples of this [Matsumara & Anathaswamy 2004, Weller 2016]. However, recent findings suggest that sunlight may have a far greater systemic effect reducing risk of some cancers, regulating hormones (such as melatonin and serotonin for cognitive function), preventing childhood myopia, (recently recognised by the World Health Organisation as an emerging health risk), and preventing metabolic disorders such as diabetes [Eppenberger et al 2020, Gorman et al 2015, Lingham et al 2020, Porojnicu et al 2007, Wacker & Holick 2013, Wright & Weller 2015]. Hence, while sunlight only interacts directly with the skin and eyes, it derives systemic effects via intermediates that may, in turn, be much more far reaching and important for many physiological functions [Gorman et al 2015, Wacker & Holick 2013, Wright & Weller 2015].

The UV-A and UV-B wavelengths are largely responsible for this phenomenon. Shorter UV-B (280-320nm) is predominantly responsible for skin carcinogenesis, directly interacting with thymine and cytosine bases within DNA deriving ‘signature’ mutations termed photolesions on contact [Ikehata & Ono 2011]. Conversely, UV-A (320-400nm) does not cause significant direct DNA damage, instead interacting with different cellular components [Ikehata & Ono 2011] such as salts situated in the skin, liberating these latter mentioned compounds as nitric oxide (NO) [Holliman et al 2017, Oplander et al 2009, Pelegrino et al 2020]. In turn, NO potentially drives many physiological processes systemically (such as cardio protection) *in vivo* [Geldenhuys et al 2014, Malone-Povolny et al 2019, Weller 2014]. In this study we examine the effect of UK sunlight exposures up to 3 standard erythema doses (SED) on NO induction, direct DNA damage and cell survival. Our work is unique in that we have chosen to use actual UK sunlight, with dose rate typical for the UK summer months and not an artificial solar simulator to carry out many *in-vitro* exposures. To the best of our knowledge, we are the first group to do this. Such data would provide useful input to the risk-benefit analysis of sunlight exposures for different demographic groups.

Materials And Methods

Cell culture. Keratinocytes (FSK), endothelial cells (FSEC) and fibroblasts (FSF) were isolated from foreskins collected from neonatal donors (approximately 5–7 days old) after routine circumcision as previously described by others [Holliman et al 2017]. Informed consent was obtained preceding collection of skin. This was conducted under the approval of the Oxford Research Ethics Committee; reference 10/H0605/1.

UK Sunlight exposure of cell culture samples. Artificial light sources such as xenon arc lamps are useful for evaluation of the effects of sunlight in biological models as they streamline experiments, subverting complications and uncertainties caused by weather and challenges of accurate exposure control. However, this is not optimal for a complete assessment of real-life sunlight exposure as it is highly unlikely that the emission of the solar simulator is fully identical to natural sunlight, both spectrally and in terms of dose rate.

Experiments in this study used exposure to actual UK sunlight on the roof of the UK Health Security Agency (UKHSA) in Chilton (51.575°, - 1.318°) from May to August 2021 under rain-free conditions, mostly on days with low cloud cover. A purpose-built temperature-controlled bench enabling thermal contact of cell plates was used to expose cells, allowing cells to be kept at a constant temperature (30^{o}C). Real-time erythema and UV-A irradiance data from a co-located UKHSA solar monitoring ground station [https://uk-air.defra.gov.uk/data/uv-index-graphs] was used to control the duration of exposure to reach the required dose. Spectral irradiance was collected by UKHSA reference double-grating DTMc300 spectroradiometer (Bentham Instruments, Reading, UK) at the same location. The Solar monitoring ground station comprises SL-501A-UV Robertson-Berger meter (SolarLight Inc, Glenside, USA) and SD-104Acos (Macam Photometrics Ltd, Livingstone, UK) sensors for erythema effective irradiance and UV-A irradiance measurements with the sampling rate of 1.2s.

UG11 solar-blind, LPW331 and LPW418 long pass filters shown in Fig. 1 were used to select UV and visible/infra-red spectral ranges, respectively; exposure to the full solar spectrum was carried out through a fused silica blank. Exposures were adjusted for filter transmittance and nominally identical exposures within each set were achieved by controlling exposure duration using real-time irradiances from the solar monitoring ground station. Full solar spectrum, UV only ($< \sim 400$nm) and visible/IR only ($> \sim 420$nm) samples were compared against an unexposed control shielded from light. To account for artefacts induced by heat, including heat due to filter absorption, additional controls were introduced to quantify heat-related contributions. Exposure doses were grouped as low (1–2 SED), medium (3–4 SED) and high ($\geq$ 5 SED) to irradiate *in vitro* primary skin cell types isolated from tissue biopsies as highlighted above; quoted doses are expressed as a dose equivalent of full spectrum irradiation.

Sol-2 Solar simulator exposure of cell culture samples. The SOL-2 solar simulator (Dr. Hönle AG UV - Technologie) with a range of bandpass filters shown in Fig. 2 was used for detailed investigation of cell survival and spectral response of NO release. Similar to sunlight exposures, duration of irradiation was

adjusted to account for spectral power distribution of illumination source and filter transmission to provide identical exposure doses of 1.9 J/cm^2. In addition to unexposed control, heat control samples were used quantifying heat-related contributions caused by filter absorption.

Nitric oxide detection in cell lines using DAF-FM DA and DAX-J2 red. DAF-FM diacetate (DAF-FM DA) and DAX-J2 RED (DAX J2) are highly specific cell permeable dyes, that unlike other quantitative methods for NO release assess NO directly, and not via by-products such as nitrite or enzymatic NO synthase (NOS). This allows only NO generated within the cell itself to be detected.

Loading of either DAF-FM DA (Molecular Probes, D23844) or DAX J2 (AAT Bio, 16301-AATB) was performed as described previously [Holliman et al, 2017]. To summarise, the compounds were used separately and were added at a 5 mM concentration in DMSO to 1 ml of culture media devoid of Phenol-red and foetal bovine serum. After addition, the compounds were incubated for 45 minutes at 37^o C prior to irradiation. Media containing either DAX-J2, or DAF FM DA were then removed, and the cells washed with HBSS-/- (SIGMA, H6648). After washing the cells were removed from the plate by Trypsin-EDTA solution. Cells were then centrifuged and re-suspended in Dulbecco PBS+/+ (SIGMA, D8662). media without phenol red and FBS were used as previous work has inferred that the compounds react poorly in the presence of phenol red and other serum-based components.

After the exposure 150 µl of the cell suspensions were then loaded in triplicate into 96-well plates. The suspensions then had 150 µl PBS with either 50 µg/ml propidium iodide (PI) (SIGMA, P4170), or SYTOX green (Thermo, R37168) added and were read with a Guava EasyCyte HT flow cytometer (Merk Millipore). Cells positive for PI or SYTOX green were excluded from further analysis. This technique was used for exposures using the SOL-2 solar simulator and actual UK sunlight.

Western blotting. FSK were grown to between 95–100% confluency in 6 cm cell culture dishes in CNT-09 media. Media was then removed, and the cells rinsed with HBSS-/-. 3 ml of pre-warmed PBS+/+ was added to the cells and the cells were exposed to sunlight. Following exposure plates were returned to the incubator, washed in HBSS-/- and then placed into fresh media. Cells were harvested at 2 hours post-exposure, as previous data suggest that gamma H2AX used to quantify DNA damage is maximally upregulated at this timepoint [Holliman et al 2017, Hazell et al 2022]. Cells were harvested and western blots run via a standard protocol on 12% separating Tris-glycine gels with a 5% stacking buffer as described previously [Hazell et al 2022]. H2AX assessment was carried out against control antibody GAPDH (1:5000) (Santa-Cruz, SC25778) to ensure correct loading. Primary antibodies were detected via chemiluminescence with donkey anti-rabbit antibody (Santa-Cruz, SC2313) at a 1:10,000 concentration following incubation for 1 hour at room temperature.

Cell survival assays. Assessment of cell survival after light exposure was performed using a cell counting kit 8 (WST-8, Abcam ab228554). The kit assesses cell survival via the action of a tetrazolium salt that when ingested by live cells is emitted as a water-soluble formazan dye, staining surrounding media orange. The degree of colour change by measuring absorbance at 460 nm is then compared with a

negative control to determine cell viability. Assays were performed at 24 hours and 48 hours post-exposure from 3 donors by addition of the cell counting kit at a 1 in 40 dilution in PBS+/+ to live cells. FSEC and FSK primary cell lines were chosen as these cells (unlike FSF) are grown as a monolayer as they are contact inhibited at confluency, hence cell numbers between wells would be approximately identical. FSK were grown in CNT-07 media and FSEC were grown in ECMV media, with light exposure being carried out when the cells reached 100% confluency. Alterations in cell survival at these time points were highlighted by carrying out the assay in triplicate, three times for each cell type and timepoint.

Ethics and regulatory approval and set-up of methods. All methods carried out as part of this project were performed in accordance with relevant guidelines and regulations. For in vitro work undertaken written consent was obtained from parents or local authority representatives was obtained prior to collection of skin samples from neonates. This was conducted under the approval of the Oxford Research Ethics Committee; reference 10/H0605/1.

Results

UV-B mediated damage within samples is negligible with ‘low-dose’ (1 SED) UK sunlight.

Any potential positive systemic effect of UK sunlight exposure has to be carefully balanced against damage that could occur in the skin, largely through UV-B exposure. Assessment of cell survival up to 48 hours after full spectrum simulated sunlight exposure suggested that cells lying within both the epidermis (FSK) and dermis (FSEC) have a good tolerance to sunlight at 1 SED, comparable to unexposed samples. However, higher exposures reduced cell survival in both cell types, with endothelial cells having a lower tolerance to sunlight overall in these instances after 48 hours. Experiments repeated on FSK with UV-B removed (using a LPW331 filter) and retaining UV-A only at 3 SED highlighted the fact that UV-B radiation in sunlight is the primary driver for cell death, reducing cell death by up to 90 % (Fig. 3.

We next assessed levels of direct DNA damage via phospho-H2AX expression, (a DNA repair enzyme and marker for assessment of double stranded DNA breaks). For this, full spectrum sunlight exposure was assessed against exposure to visible/infrared light and UV light only using UG11 and LPW filters (Fig. 4). FSKs were selected for these experiments as the cell forms 90% of the skin’s upper dermis, and also as cancers with highest incidence rate linked to sunlight exposure originate in this cell line [Marionnet et al 2014]. Evaluation of results highlighted that UV radiation conferred all direct DNA damage on FSK and was directly proportional to dose applied. Interestingly, when the UV spectra was isolated and used alone, it appeared to confer greater H2AX upregulation than full spectrum irradiance for the equivalent erythema dose. We also noted that the H2AX upregulation in samples irradiated by the lowest dose of 1 SED of full spectrum sunlight was comparable to unexposed controls. This re-iterated conclusion that low dose exposures to full spectrum UK sunlight confers insignificant adverse effects to skin cells *in vitro*.

Low dose sunlight at 1 SED induces nitric oxide readily in all skin cells.

The fact that low dose sunlight exposure doesn't increase cell death and derives little elevation in DNA damage is very important if this level of exposure is sufficient to induce NO production (Fig. 5) through a UV-A route. In addition to FSK, we assessed this effect in FSEC and FSF skin cells situated deeper within the skin's dermis. Even at the lowest doses (equivalent to 1 SED of full spectra irradiation) we found NO induction to occur in all skin cell types assayed; increasing doses of sunlight potentiated this effect, with FSK giving the lowest overall yield of NO, FSEC deriving the highest yields at low doses and FSF deriving highest yields at higher doses (Fig. 5).

DNA damage and cell death assays suggested that adverse effects were directly proportional to increased sunlight exposures. This trend was also seen in nitric oxide induction for keratinocytes, although the trend was not significant in all cases (bar 15- and 30-minute exposures) when each nearest higher exposure was considered (Fig. 6).

Shorter wavelength UV-A appears to be more efficient in sunlight-induced NO generation, from nitrite and the main mediator for effect seen, but whole UV-A spectrum is capable of NO generation.

Initial results with UK sunlight exposure in an *in vitro* cell culture model suggested that the use of DAF-FM diacetate may be hindered by visible light. This was evident as UV-alone produced a marked upregulation of NO but when samples were exposed to full spectrum UK sunlight or visible light only they appeared to produce less NO than the unexposed control (See supplementary information). Evaluation of this phenomenon suggested that DAF-FM DA photobleaching by visible light may be responsible for this effect. We hypothesised that this occurred due to the fact that excitation spectra of DAF-FM peaks at 495 nm, thereby full spectrum and visible light (> 420 nm) exposure induced photobleaching prior to quantitative analysis. To avoid this experimental artefact, a different compound, DAX-J2 red with excitation in the red spectral range, was used for experiments with simulated sunlight filtered by UV-A bandpass filters (shown in Fig. 2). Use of DAX-J2 with FSK and FSEC cells suggested that the shorter UV-A wavelengths are considerably more efficient in NO production, consistent with results by others [Pelegrino et al 2020]; however, exposure to longer UV-A wavelengths also resulted in NO induction with lower efficacy (Fig. 7). This work matched previous findings suggesting that nitrite with a peak absorption in 340–360 nm is the main derivative broken down by UV-A in sunlight to NO [Hazell et al 2022, Holliman et al 2017, Liu et al 2014, Oplander & Suschek 2012,].

Discussion

UV wavelengths within sunlight elicit DNA damage at different rates within skin cells, with UV-B up to 10,000-fold more damaging than longer UV-A [Holliman et al 2017]. In line with this thinking *in vitro* research demonstrates epidermal skin cells tolerate low level UV-A irradiation 7–9 J/cm^2 (equivalent to 'sub-erythemal' doses of full spectrum sunlight) more effectively than UV-B [Marionett et al 2014]. Our work assessing exposures lower than 2 SED (that others claim do not provoke noticeable erythema in fair skinned Fitzpatrick type I individuals, [Harrison et al 2002]) substantiate these claims, as in these instances 1 SED UK sunlight elicited little DNA damage in epidermal and dermal cell lines. However, at

higher exposures (at or above 2 SED) a difference in cell survival was prevalent 48 hours after exposure, with keratinocyte survival up to 50% higher compared with endothelial cell lines.

These results bring to the fore findings by D'Errico et al and others who suggest skin cells tolerate sunlight exposure in a diverse manner, with 'deeper' dermal cells more susceptible to negative effects of sunlight than epidermal cells [D'Errico et al 2005, Goyeneche 2020]. As keratinocytes form 90% of the skin's epidermis providing the physical barrier between toxic insults within our environment, this line of thinking is indeed feasible [Nestle 2009], however, as D'Errico's work utilized fibroblasts as the dermal cell type, (suggesting a higher propensity for keratinocytes to remove UV induced signature mutations). Further work is needed to confirm if this effect transcends to endothelial cell lines and other dermal cell types.

Although our own *in vitro* work showed this effect (where cells are simply present as a monolayer), differences in DNA damage tolerance between epidermal and dermal skin cells *in vivo* may in fact be much smaller through attenuation of short wavelength UV-B that we understand is attenuated more effectively than other longer wavelengths [Finlayson et al 2021]. Using this model and the UK summer solar spectrum, we estimated that 1 SED incident on the skin surface is attenuated to 0.0047 SED on the top of the dermis, to 0.0002 on the top of the basal layer and drops below 0.0001 in the dermis. Therefore, skin attenuation should indeed be taken into account when comparing damage tolerance of basal and dermal cells. Similarly, UV-A in sunlight is also attenuated by the skin, though to a lesser degree: to 0.069 when reaching the epidermis, 0.006–0.004 in the basal layer and drops below 0.0033 in the dermis. Our results therefore suggest longer wavelengths within the UV-A spectra reaching these deeper dermal cells could give rise to greater quantities of NO than in FSK (in which this trend is mostly associated with). This data backs up our earlier work which points out that UV-A can induce NO production in multiple skin cell lines for a considerable amount of time after exposure and is derived not only from the breakdown of salts [Hazell et al 2022].

Although UV-B in sunlight is responsible for the majority of negative effects on the skin at high dose, these wavelengths constitute less than 5% of sunlight's spectrum [Kricker et al, 1994]; in comparison, visible and infra-red radiation is 10-fold more predominant [Kricker et al, 1994]. Contribution of these longer wavelengths in sunlight to skin damage was investigated in this study, either on their own or in conjunction with UV. No evidence of damage in skin cells by sunlight's visible or infrared radiation at equivalent sub-erythemal and erythemal doses was found; instead, full spectrum sunlight exposure showed a trend towards lower fold changes in the DNA damage marker gamma H2AX in FSK compared with UV irradiation alone. This finding validates work by Barolet et al [Barolet 2021, Barolet & Christians 2015], who suggest that low levels of infra-red radiation within sunlight play a role in mitigating UV damage through DNA damage repair during the morning and evening when UV levels are low but infra-red and visible light remain high. It is therefore feasible that moderate sunlight exposure may promote protective responses in the skin.

Finally, although use of actual UK sunlight may mitigate variability of results seen between some other studies due to use of different artificial light sources where spectral power distribution of irradiation varies, other caveats and limitations of an *in-vitro* set-up need to be considered. Firstly, as others have suggested repeated low dose exposures (from 7–9 J/cm^2) administered over an extended timeframe potentially derive adverse effects, not witnessed through 'one-off' irradiation [Seite et al 2010]. Secondly, Fitzpatrick scale (lost within an *in-vitro* model) may play a role *in-vivo*, potentially altering DNA damage long after the event via induction of 'dark cyclobutene pyrimidine dimers (CPDs)' and through the protective effect of melanin changing the threshold of direct DNA damage [Fajuyigbe et al 2020, Supp et al 2020] seen in our studies. Lastly – and perhaps most importantly, is the complexity of the NO pathway *in vivo* with age, where reduced bioavailability of NO occurring through loss of function within the nitric oxide synthase enzyme (NOS) rather unfortunately gives rise to toxic reactive oxygen species superoxide and peroxynitrite, further compromising health status in these individuals. Hence, to truly feed into advice for healthy sunlight exposure we need to be mindful of these additional factors *in vivo*.

To Conclude

Our work suggests that *in vitro* epidermal and dermal skin cell lines produce the potent cardio-protectant NO under low dose UK sunlight (1 SED). Remarkably, this low-level exposure does not negatively affect cell survival and DNA damage witnessed at higher doses of sunlight, and we postulate that longer wavelengths outside of the UV solar spectrum may play a role in mitigating adverse effects. In line with others, it was shown that UV-A within the solar spectrum is the largest donor for NO induction, with a maximum of nitrite breakdown between 340–360 nm and effective to generate NO up to 400 nm. Although these results support claims that low dose sunlight may *in vivo* provide cardiovascular benefit to specific demographics such as the elderly or those at higher risk of cardiovascular disease further work *in-vivo* is needed to validate these claims. However, these data provide a useful insight to further research on sunlight exposure guidance for different demographics.

Declarations

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Data availability statement: Please note, the datasets used and analysed in this study are available from the corresponding author on reasonable request.

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Supplementary Information

The Supplementary Information is not available with this version

Figures

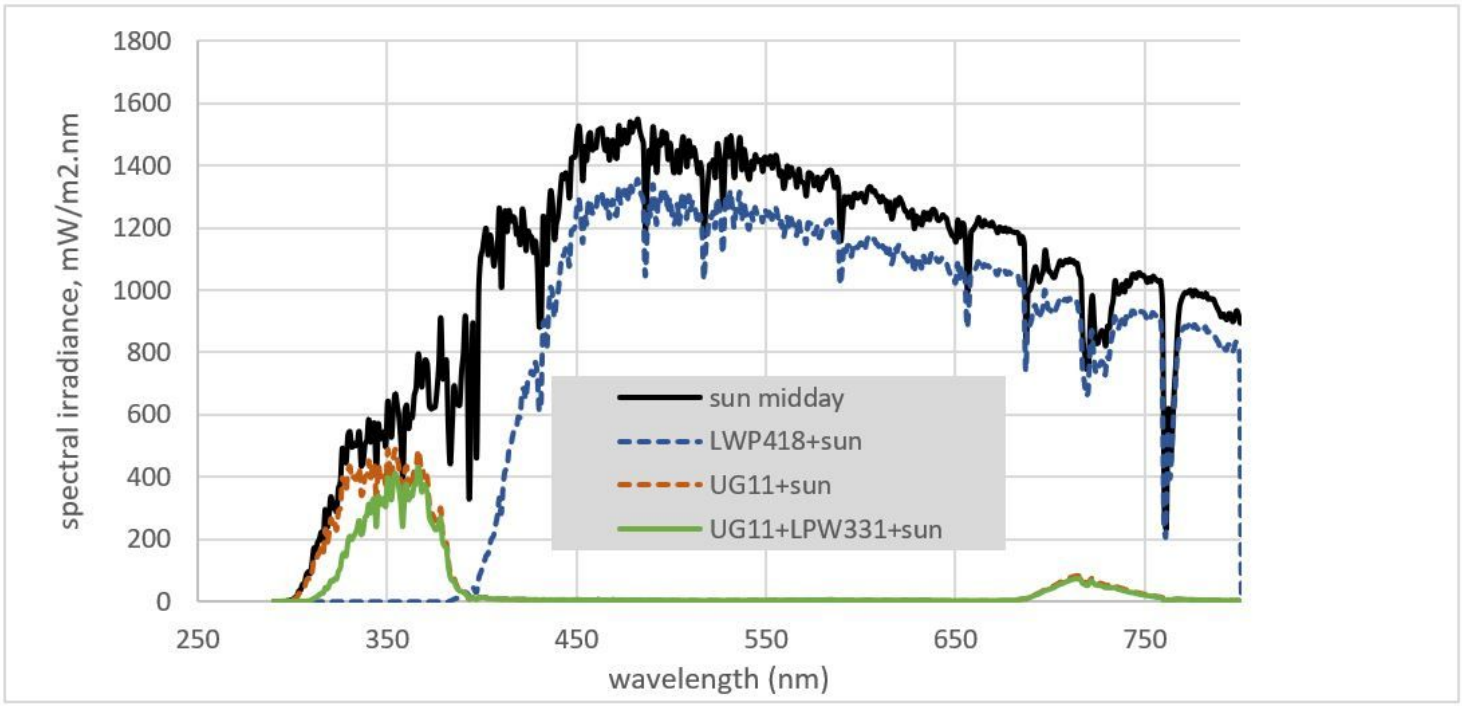

Figure 1

Spectral irradiance of sunlight at midday in Chilton and sun filtered by filters.

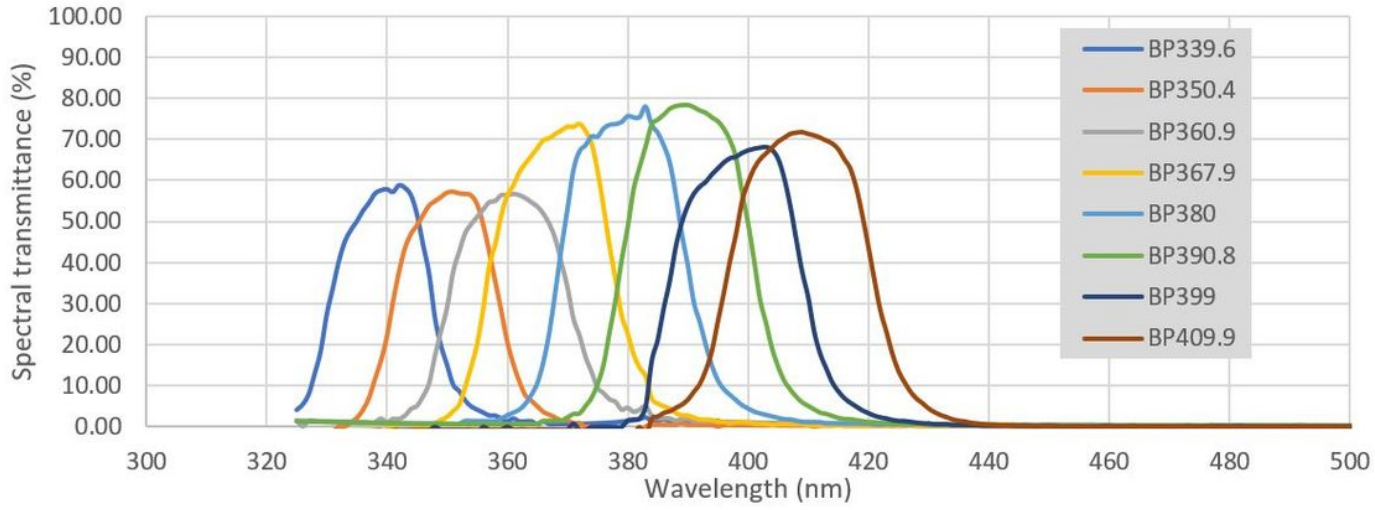

Figure 2

Transmittance of filters used to evaluate UV-A spectral response of nitric oxide release

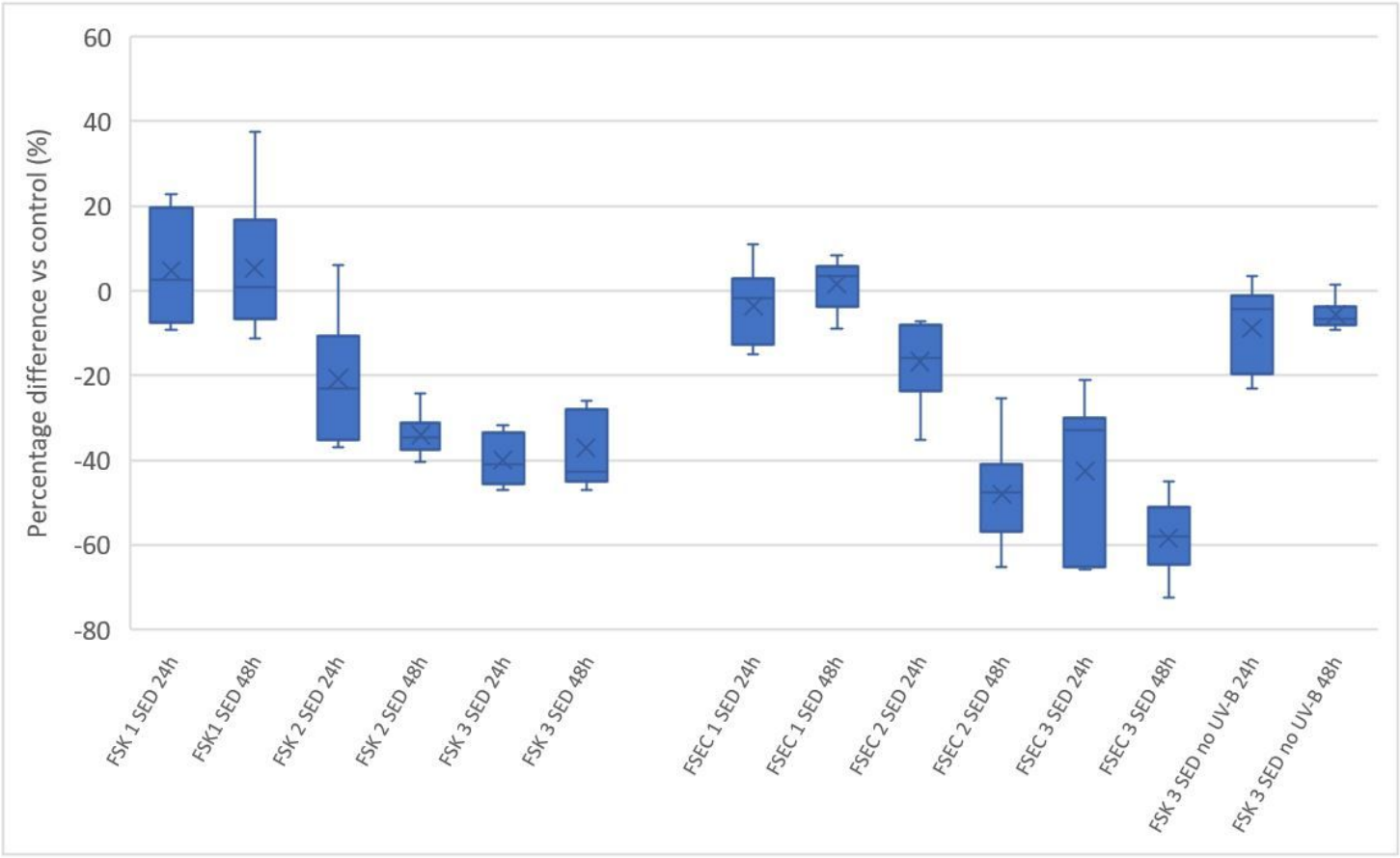

Figure 3

Three experiments carried out in triplicate using neonatal foreskin keratinocyte donor Cell lines (FSK) and neonatal foreskin endothelial cell lines (FSEC). Cells were exposed to 1, 2 and 3 SED solar simulated sunlight and cell viability was recorded 24 and 48 hours after exposure with use of the CCK-8 assay. 1 SED derived on average the same cell viability as the unexposed control. Higher exposures derived significantly lower yields in cell viability in comparison, with a dose dependent drop in cell viability. Further assessment with removal of UV-B wavelengths suggested with effect could be largely attributed to UV-B.

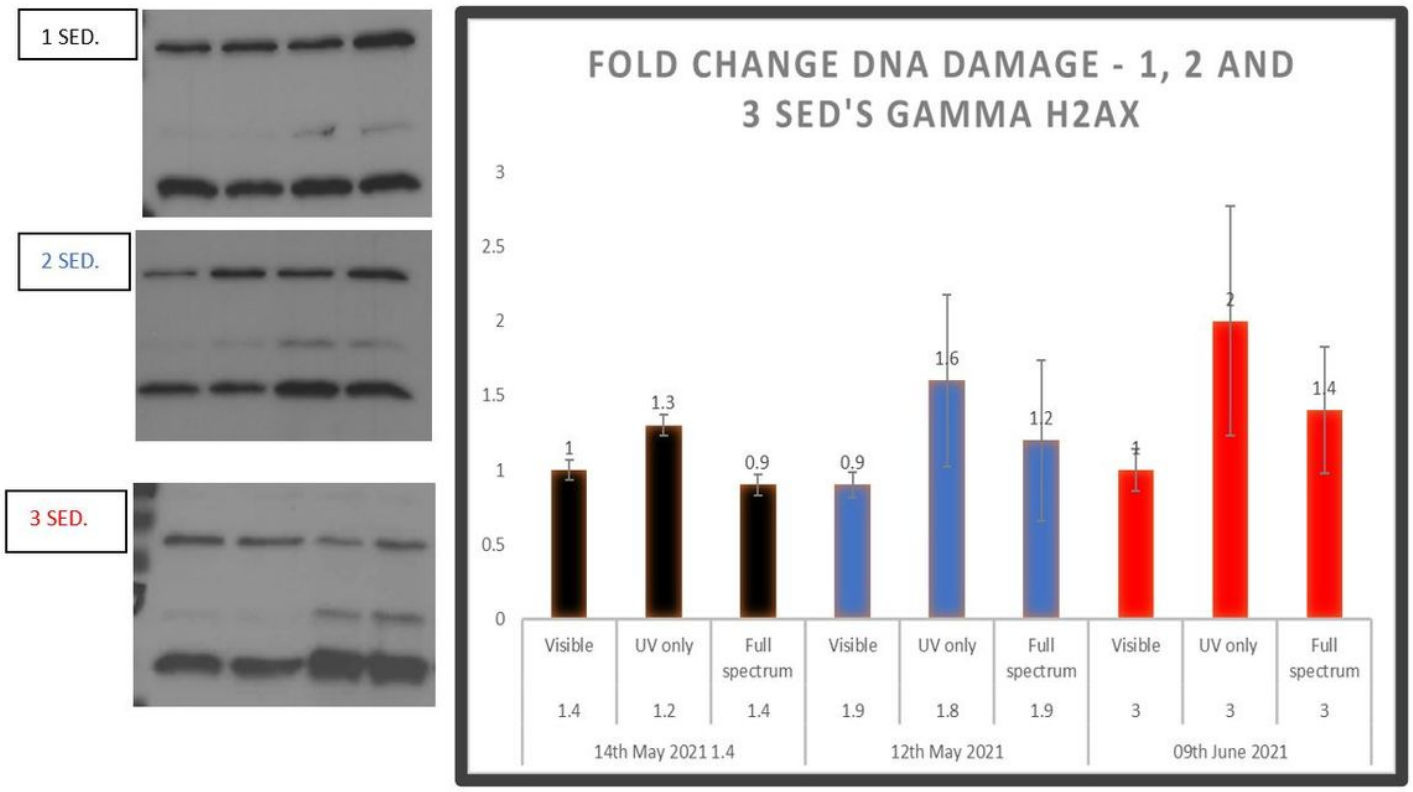

Figure 4

Western blotting of gamma H2AX in FSKs exposed to 1 SED (black), 2 SED (blue) or 3 SED (red) of sunlight. Exposures are quoted in the doses equivalent to full spectrum exposure and were carried out on the roof of the UKHSA in summer months. Visible light derives no negative effect on H2AX and the damage is attributed to UV exposure. At low doses (1 SED) negative effects on keratinocytes were negligible.

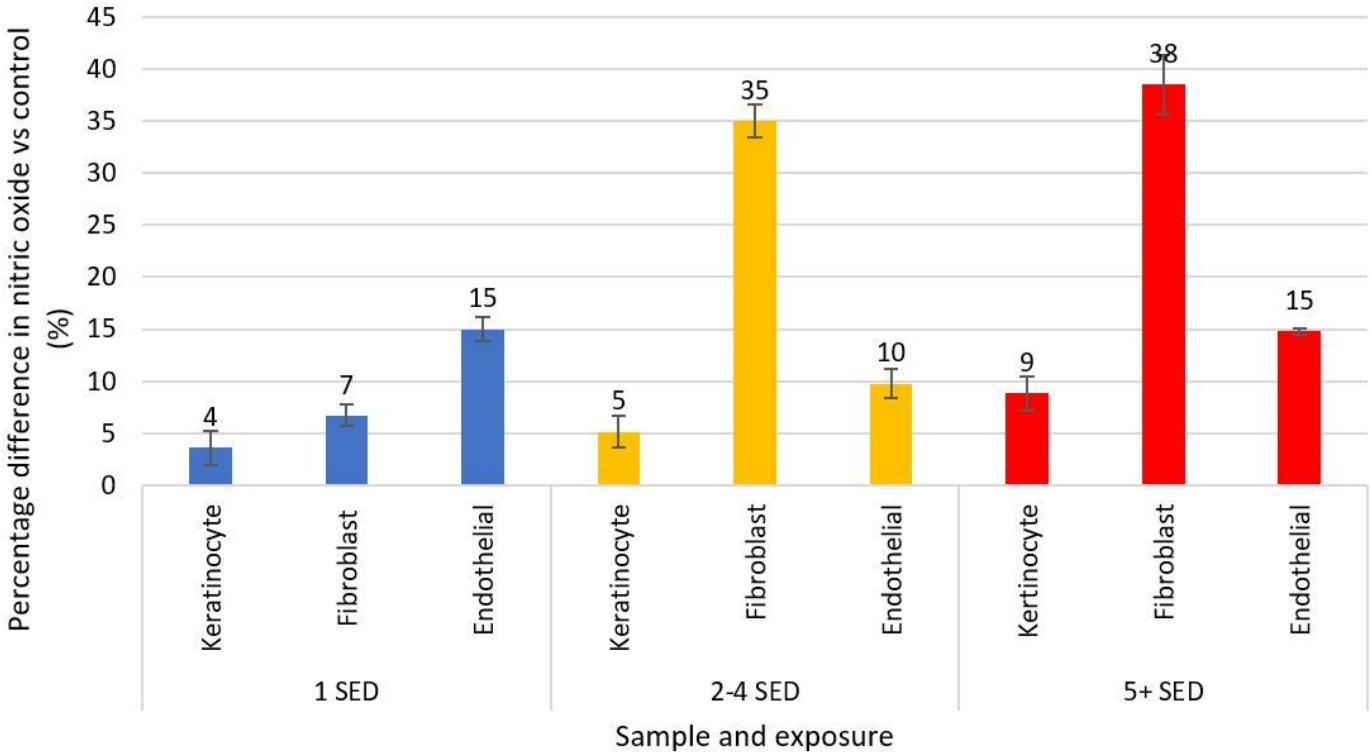

Figure 5

Nitric oxide production in keratinocyte, endothelial and fibroblasts skin cells following exposure to increasing doses of UK summers sunlight. It was noted that a low dose (1SED) of UK summers sunlight (that derived little damaging effects in endothelial and keratinocyte skin cells *in vitro*) was capable of giving rise to nitric oxide in all skin cells assessed.

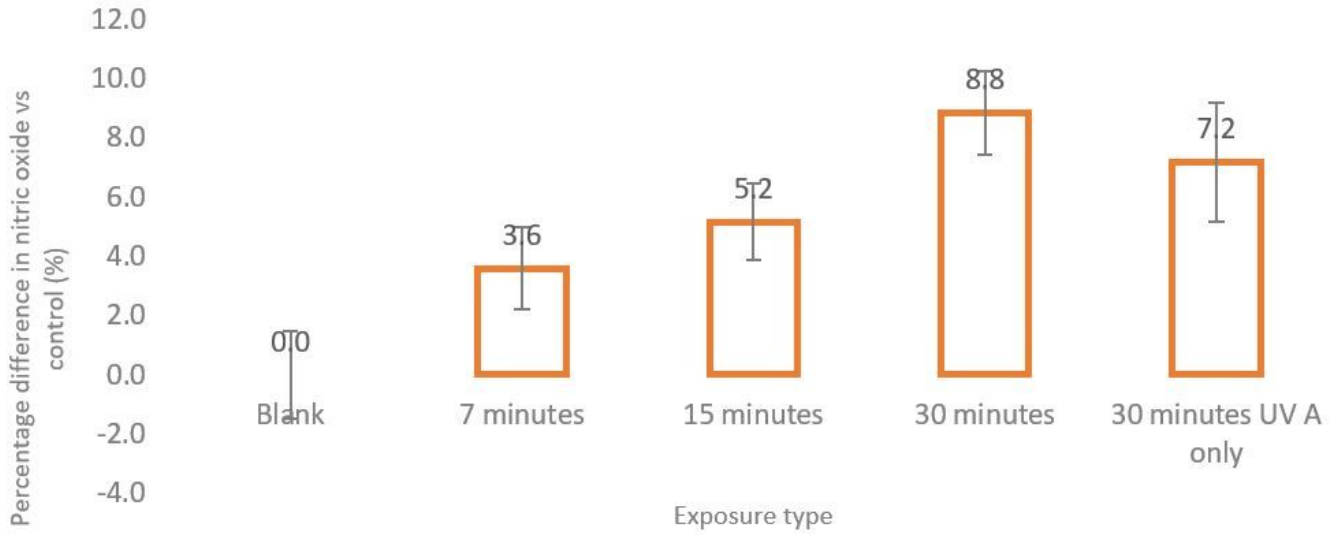

Figure 6

Dose response in keratinocytes at increased timescales of solar irradiance,

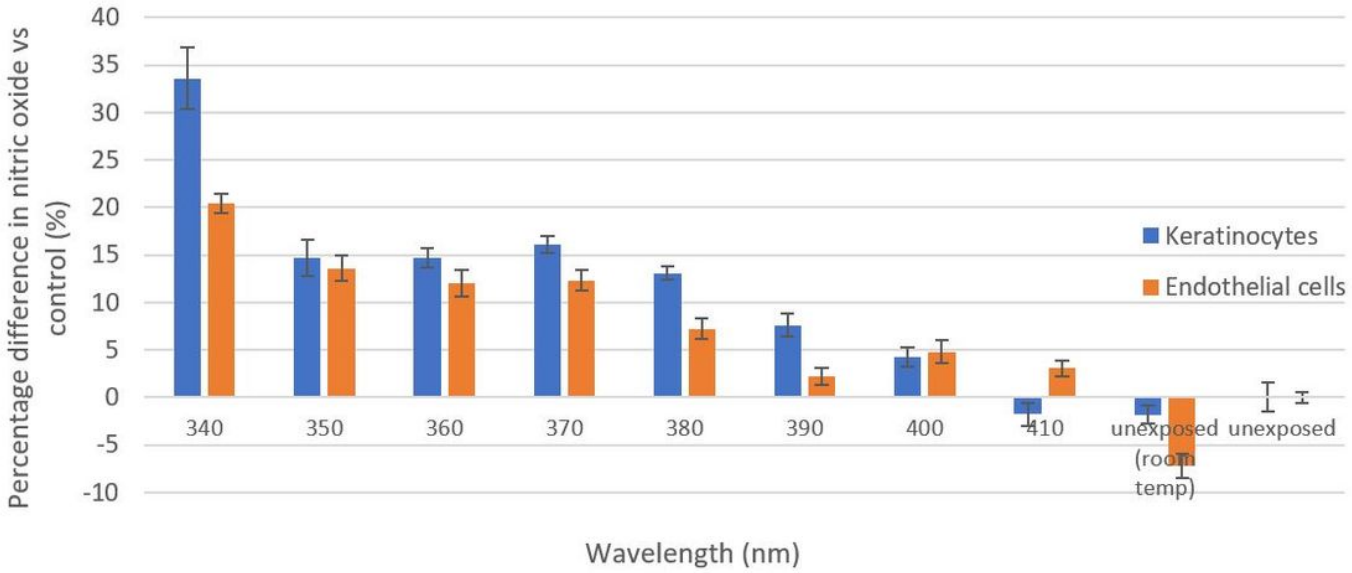

Figure 7

Production of nitric oxide from endothelial and keratinocyte skin cells after exposure to filtered simulated sunlight. Exposures were adjusted for filter transmission to produce 1.9 J/cm^2.