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Medium Mediated Effects Increase cell Killing in a Human Keratinocyte Cell Line Exposed to Solar Simulated Radiation

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Title

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Abstract

Purpose: The objective of this study is to investigate whether cell culture medium is a biologically relevant exposure medium that can be employed in non-ionising photobiological investigations.

Methods: The effect of solar simulated irradiation on cell culture medium and its ability to elicit cell death was studied. The role of reactive oxygen species (ROS), cell secreted factors, and the contribution of individual components of the medium were investigated.

Results: Cell death was found to be primarily mediated through the formation of ROS via riboflavin photosensitisation and degradation in the cell culture medium. Phenol red was found to significantly reduce the cell killing ability of riboflavin. Exposures in riboflavin free medium resulted in significantly increased cell survival compared to identical exposures in riboflavin containing medium.

Conclusions: This study has shown that solar radiation toxicity is augmented by cell culture medium due to the presence of riboflavin. Results suggest that exposures performed in phenol red free medium may serve to increase phototoxic effects if riboflavin is present. Riboflavin free media is recommended for solar radiation investigations to eliminate concerns regarding riboflavin photosensitisation and nutrient deprivation.

Introduction

The incidence of skin cancer is increasing and although solar ultraviolet (UV) radiation is known to be the main environmental risk factor (Assefa et al. 2005), the mechanisms through which radiation interacts with cellular processes are still poorly understood. This lack of understanding is, in part, due to the fact that in vitro studies employ different cell models, end points and, most fundamentally, different exposure parameters making laboratory inter comparisons of results difficult. Two such parameters are the radiation source and exposure media employed.

Non ionising photobiological exposures are predominantly performed in either cell culture media or buffers such as phosphate buffered saline (PBS) however, rarely is the reasoning behind the choice of exposure medium specified. Previously, during a reciprocity study examining the effects of solar simulated radiation on HaCaT cells, an immortalised human keratinocyte cell line, we found significant differences in survival post irradiation when cells were irradiated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium (DMEM-F12; Sigma, Dorset, U.K) and PBS (Maguire et al. 2010). Despite significantly reduced transmissive properties, DMEM-F12 medium was found to be far more phototoxic than PBS. Furthermore, this effect was observed to be wavelength dependent.

These surprising results spurred a more in depth analysis to determine how DMEM-F12 medium accentuates cell death. The role of ROS, the role of radiation induced cellular secretions and the effects of irradiation on DMEM-F12 itself and its components were all investigated.

Materials and Methods

Cell culture and seeding

HaCaT cells (a kind gift from Dr Petra Boukamp, DKFZ, Germany), are immortal but non-malignant with a doubling time of 21 hours (Boukamp et al. 1988) and mutations in both p53 alleles (Lehman et al. 1993). HaCaT cells were cultured in DMEM-F12 cell culture medium containing 10% fetal bovine serum (FBS, Gibco, Irvine, U.K.), 1% penicillin-streptomycin solution 1000IU (Gibco) and 1 μg/ml hydrocortisone (Sigma). Cells were incubated under humid conditions at 37ºC, with 5% CO_2 in air. Subculture was routinely performed when cells were 80-90% confluent, using a 1:1 solution of 0.2% trypsin (Sigma) and 1 mM versene (Sigma) at 37ºC. A single cell solution was generated in fresh DMEM-F12 and cell counts were determined using a haemocytometer. HaCaT cells were determined to have a plating efficiency of 27.2 ± 3.6%, thus, in order to obtain a reasonable number of colonies, cells were seeded at 400 cells in 3 ml DMEM-F12 per well in Nunclon 6 well plates (Biosciences, Dublin, Ireland) and incubated (conditions as above) overnight (16 hours) prior to irradiation. The extended incubation period between seeding and irradiation was employed for all experiments since PBS exposures required cell washes which resulted in reduced and irregular control colony numbers compared to non-washed controls when sufficient time for attachment was not allowed. However, dishes were checked prior to irradiation to ensure that the plated cells had not divided and that they were still single cells.

Dosimetry

All irradiation experiments in this study were performed using the Q-sun solar simulator (Q-panel, Cleveland, Ohio, USA). The Q-sun was calibrated using spectroradiometry which is described in detail elsewhere (Maguire et al. 2010). In brief, a miniature fibre optic USB2000 spectrometer (Ocean Optics, Duiven, The Netherlands) with a response over 200-1100 nm was calibrated using a Bentham CL6h spectral irradiance standard (Bentham instruments Ltd, Berkshire, UK) which has a spectral irradiance from 250 to 3000 nm and is traceable to National Physical Laboratory (NPL) standards (NPL, Teddington, UK). Incident radiation is transmitted to the spectrometer via a 600μm optic fibre and cosine corrected using a CC-3-UV PTFE cosine diffuser (Ocean Optics). Once calibrated, the spectrometer was employed to obtain the calibrated spectral distribution in Wm^{-2} at the exposure level of the Q-sun solar simulator which is shown in figure 1. Integrating the spectral distribution from 280 nm to 400 nm yielded a total UV intensity of 63.63 Wm^{-2}, 62.3 Wm^{-2} in the UVA (315-400 nm) and 1.33 Wm^{-2} in the UVB (280-315 nm) region. Exposures are presented in terms of time but can be converted to energy using the fact that 1 Wm^{-2} equals 1 $Jm^{-2}s^{-1}$. For example a 10 minute (600 second) exposure at an intensity of 63.6 Wm^{-2} (63.6 $Jm^{-2}s^{-1}$) provides a dose of 38,160 (63.6 x 600) Jm^{-2} or 3.816 Jcm^{-2}.

Exposures

Once ignited, the Q-sun was allowed to stabilise for a minimum of 15 minutes. Immediately before each irradiation, the exposure field of the Q-sun was sterilised using 100 % methanol which allows exposures to be performed without lids thus eliminating attenuation effects due to the plastic lid. Cells were irradiated for 2, 5 or 10 minutes in DMEM-F12 or 10, 30 or 60 minutes in PBS where a 2, 5, 10, 30 or 60 minute exposure corresponds to a UVA/B dose of 0.8, 1.9, 3.8, 11.4 and 22.9 Jcm^{-2} respectively. Post exposure, the cells removed from the exposure field and returned to the incubator for 7 days before survival was assayed using the clonogenic assay. Controls were handled identically but received sham irradiation. Secondary controls termed 'incubator controls' were also included for each experiment to determine the effects, if any, of handling and antioxidant supplementation on the primary controls. No difference in survival was observed due to handling or antioxidant supplementation.

Direct DMEM-F12 exposures;

Cells were seeded and exposed as outlined above with no medium change before or after exposures.

Direct DMEM-F12 exposures with antioxidants;

Cells were seeded as outlined above. Before irradiation, cells were removed from the incubator and supplemented with an antioxidant. Antioxidant final concentrations of 20 μg/ml, 100 μg/ml and 5 mM in 3 ml DMEM-F12 for catalase (Sigma, EC 1.11.1.6), superoxide dismutase (SOD, Sigma, EC 1.15.1.1) and reduced glutathione (GSH, Sigma, EC 200-725-4) respectively were chosen based on values used in the literature (Dahle et al. 2005, Lyng et al. 2006). While a non-lethal concentration of 0.5 % v/v dimethyl sulfoxide (DMSO, Sigma) was pre-determined from dose response experiments performed (data not shown).

Blank DMEM-F12 medium transfers;

Pre warmed (37°C) blank DMEM-F12 medium (no cells) supplemented or not supplemented with antioxidants at the above final concentrations was exposed in 6-well plates at a volume of 3 ml per well. Post exposure, the irradiated blank media ± antioxidant was harvested, filtered and transferred to unirradiated recipient single cells immediately, 1 hour, 24 hours and 48 hours post exposure. Irradiated media for the 1, 24 and 48 hour transfer time points were incubated post exposure until the appropriate time at which each was harvested, filtered and transferred to recipient single cells. Recipient cells were seeded, as outlined above, 16 hours before receiving irradiated blank DMEM-F12 ± antioxidant. DMEM-F12 covering the recipient single cells was discarded and after transfer of irradiated blank DMEM-F12 ± antioxidant, recipient cells were returned to the incubator for 7 days.

Donor cell DMEM-F12 medium transfers;

Donor cells were seeded such that at the time of transfer post exposure densities of 0, 1 x 10^5, 2 x 10^5, 5 x 10^5 and 1 x 10^6 cells would be expected. Donor cells were irradiated as outlined above in 3 ml of DMEM-F12 and returned to the incubator immediately post exposure. Irradiated donor cell medium was harvested, filtered and transferred to unirradiated recipient cells immediately, 1 hour, 24 and 48 hours post exposure. Recipient cells were seeded, as outlined above, 16 hours before medium transfer. The DMEM-F12 covering recipient cells was discarded and the donor cell medium was transferred from the donor cells to the recipient cells after which the recipient cells were returned to the incubator for 7 days.

The irradiated DMEM-F12 medium was filtered prior to transfer using 0.2 μm filter (Nalgene/Thermo Fisher Scientific, Roskilde, Denmark) to ensure that unexposed recipient cells were treated with cell free supernatant from irradiated donor cells. Although cells were not present during blank medium irradiations, the medium was also filtered to facilitate inter-comparisons of the donor cell and blank medium transfers.

Direct PBS (with and without reagents) or L-15 exposures;

Cells were seeded and exposed as outlined above. However prior to exposure, DMEM-F12 was removed from the cells, filtered and stored in the incubator. Cells were washed once with 1 ml pre warmed (37°C) PBS and the wash discarded. Cells were covered in 3 ml fresh pre warmed exposure medium (PBS, phenol red (0.00863 g/l, Sigma) in PBS, phenol red (0.00863 g/l) and riboflavin (0.00022 g/l, Sigma) together in PBS, riboflavin (0.00022 g/l) in PBS or Leibovitz riboflavin free cell culture medium (L-15, Sigma) containing 10% FBS, 2 mM L-glutamine (Sigma), 1% penicillin-streptomycin and 1 μg/ml hydrocortisone) and then exposed. Post exposure, the exposure media was discarded and the stored DMEM-F12 replaced at 3 ml per well before the cells returned to the incubator for 7 days. All post exposure medium changes were carried out immediately post exposure except for L-15 exposures which were changed 4 hours post exposure.

Clonogenic assay

Clonogenic expansion of single cells was determined using a method devised elsewhere (Puck and Marcus 1956). This method allows survival to be quantified post exposure to some toxic event by the ability of single cells to form macroscopic colonies distinguishable from one another, where such a colony is said to be a group of approximately 50 or more cells which should arise from a single cell.

With a doubling time of 21 hours, a 7 day incubation period is sufficient for HaCaT cells to form macroscopic colonies. Thus following treatment, cells were incubated for 7 days, after which cells were stained using a 20 % carbol fuchsin solution for 5 minutes and scored. Survival curves were determined from the data collected.

Statistics

Results represent 3 independent tests, normalised to controls and presented as the mean +/- the standard deviation. Statistical analyses of variance (ANOVA) were performed on the linear regressions and pair-wise data using the Bonferroni adjustment were obtained. All analyses were done using statistical software package SAS 9.1 and SAS enterprise guide 3.0. Significance was taken at a level of $p \leq 0.05$.

Results

Figure 2 shows the survival of HaCaT cells directly exposed in DMEM-F12 medium with and without the presence of antioxidants. Cells directly exposed in the presence of SOD and DMSO showed no significant difference compared to cells exposed without antioxidants, indicating that neither superoxide nor hydroxyl radicals play a significant role in solar radiation induced cell death in DMEM-F12 medium. Survival was significantly increased in cells exposed directly in DMEM-F12 medium supplemented with GSH and catalase compared to cells directly exposed for the same duration in DMEM-F12 medium without antioxidant supplementation. Since catalase is not cell membrane permeable (Dahle et al. 2005) this suggested a significant role for extracellular hydrogen peroxide mediating solar simulated radiation induced cell death in DMEM-F12 medium. Thus, the effect of irradiating DMEM-F12 alone was investigated.

Figure 3 shows the survival of unexposed recipient HaCaT cells treated with irradiated blank DMEM-F12 medium. Increased cell death with increasing exposure time of blank DMEM-F12 was observed, surprisingly, at all times of transfer. However, only blank DMEM-F12 medium exposed for 10 minutes resulted in a significant decrease in survival of recipient cells at all transfer times compared to their respective controls.

To establish if ROS contribute to the decrease in recipient cell survival observed in figure 3, irradiated blank DMEM-F12 medium transfer experiments were repeated with and without the presence of antioxidants. Figure 4 illustrates the survival of unexposed recipient cells treated with irradiated or sham irradiated blank DMEM-F12 medium with and without antioxidants immediately post exposure. As expected, the presence of DMSO conferred no protection, while unexposed recipient cells treated with blank DMEM-F12 medium supplemented with GSH or catalase showed significant increases in survival.

Although generation of ROS is known to be rapid and short lived, irradiated blank DMEM-F12 medium transfers with and without antioxidants were also performed 1, 24 and 48 hours post exposure (data not shown). The rationale for carrying out blank irradiated DMEM-F12 transfers in the presence of antioxidants at later time intervals was based on the supposition that the reduced survival observed at the later times of transfer in figure 3 may be due to ROS induced damage of one or more of the components of DMEM-F12 medium at the time of irradiation. The results revealed that the presence of DMSO and catalase did not have an effect on survival however GSH supplementation produced increased survival for all exposure times (2, 5 and 10 minutes) at all times of transfer (1, 24 and 48 hours).

To investigate the involvement of cell secreted factors, donor cell medium transfer experiments were performed. Figure 5 illustrates the survival of unexposed recipient cells treated with donor cell medium immediately post exposure, where a clear and significant decrease in recipient cell survival with increasing donor cell density was observed. No significant difference was found between control recipient cells despite receiving sham irradiated DMEM-F12 medium from differing donor cell densities thus eliminating reservations regarding nutrient consumption. In addition to significant differences

between all 10 minute exposures and their respective controls, all recipient cells treated with exposed donor cell DMEM-F12 medium were found to be significantly different from one another except the 1 x 10^5 donor cell density which was not significantly different from the 0 and 2 x 10^5 cell densities. These results show that the presence of cells during irradiation accentuates medium mediated cell killing effects of recipient cells in a donor cell density dependent manner. However, this effect was found to be transient, lasting less than 1 hour, since donor cell DMEM-F12 medium transfers carried out 1, 24 and 48 hours post exposure were found to elicit survival not significantly different from controls at all times of transfer (data not shown).

To investigate the effect(s) of solar simulated radiation on DMEM-F12 and its components further, fluorescence spectroscopy was employed to assess the possibility of radiation induced structural changes at the molecular level that may in turn compromise the functionality of a component in terms of nutrient value. Prior to fluorescence spectroscopy, absorption spectroscopy was employed to determine appropriate excitation wavelengths. In order to simplify presentation and avoid log-transformed data, the absorption data are presented as transmittance spectra. Figure 6 shows the transmittance spectra for select components of DMEM-F12 cell culture medium. It can be seen that DMEM-F12 absorbs significantly below 600 nm with transmission minima observed at 410 nm and 558 nm. Analysis of the components of DMEM-F12 reveals how the transmittance spectrum of DMEM-F12 is an amalgamation of the transmittance properties of its components, particularly phenol red and FBS. At the same concentration found in DMEM-F12, phenol red in PBS can be seen to have maximal absorption, observed as troughs in the transmittance spectrum, at 435 nm and 558 nm which coincides with the absorption troughs observed with DMEM-F12 but absent in phenol red free DMEM-F12 with and without 10% FBS. However, the maximal absorption of DMEM-F12 in the blue region occurs at 410 nm and not 435 nm as phenol red would suggest. This discrepancy in maximal absorption is due to the presence of 10% FBS in DMEM-F12, as verified by the transmittance spectra of undiluted FBS and phenol red free DMEM-F12 containing 10% FBS, both of which can be seen to possess absorption troughs at 410 nm as for DMEM-F12. Interestingly, the transmittance spectrum for riboflavin in PBS shows that at a concentration analogous to that found in DMEM-F12, riboflavin has minimal absorption but still retains absorption troughs at 370 nm and 440 nm as shown in the inset of figure 6, which concurs with values listed in the literature (Ali and Naseem 2002).

Based on the transmittance spectra shown in figure 6, excitation wavelengths of 370 nm, 410 nm, 440 nm and 558 nm were chosen to perform fluorescence spectroscopy on all eight solutions. Solutions were irradiated for 10 minutes in the Q-sun solar simulator and compared to unexposed solutions examined under identical conditions. Although fluorescence spectroscopy was performed using excitation wavelengths of 370 nm, 410 nm, 440 nm and 558 nm, the emission spectra obtained using an excitation wavelength of 370 nm was found to provide the most detailed account of changes incurred to irradiated media, hence only these emission spectra are presented and discussed here.

Figure 7A shows the emission spectra of irradiated and unirradiated DMEM-F12 medium with 10% FBS. Similar to the transmittance spectrum of DMEM-F12, the emission spectrum for unexposed DMEM-F12 medium can be primarily attributed to the presence of FBS and phenol red. Peak emission wavelengths for DMEM-F12 medium were observed at 450 nm, 508 nm and 584 nm. Emission at 584 nm is attributed to the presence of phenol red where irradiation of DMEM-F12 medium yields a reduction in intensity at 584 nm similar to that observed for phenol red in PBS when irradiated as shown in figure 7C. The emission peaks at 450 nm and 508 nm are attributed to the presence of FBS where irradiation of DMEM-F12 yields a loss of the peak at 508 nm and an increase in emission at 450 nm which is similar to the effect observed when undiluted FBS is irradiated as shown in figure 7B. However, when undiluted FBS is irradiated the peak emission is redistributed from 448 nm to 458 nm and may be the result of re-absorption processes (Dalton et al. 2001, Sakuma et al. 2007) and /or radiation induced structural changes to the emitting fluorophore(s) present in FBS. This effect is not observed when DMEM-F12 is irradiated and may be due to the dilution of FBS in DMEM-F12 and / or the presence of phenol red.

Comparing the emission spectra of three unirradiated solutions with and without phenol red shows the presence of phenol red to reduce the fluorescence intensity of all three solutions. The emission spectra for unirradiated PBS with and without phenol red are shown in figures 7C and 7G. The fluorescence spectrum of PBS without phenol red (figure 7G) shows PBS to have an emission peak at 420 nm despite PBS being minimally absorbing as previously shown (Maguire et al. 2010). In the presence of phenol red, the emission peak of unirradiated PBS is still visible at 420 nm (figure 7C). However, the intensity can be seen to be reduced by 70-75% due to the presence of phenol red. Similarly for DMEM-F12, the unirradiated emission spectra of the cell culture medium with and without phenol red presented in figures 7A and 7F show the presence of phenol red to reduce the peak emission intensity by approximately 25%. The third solution examined was riboflavin in PBS with and without phenol red, shown in figures 7E and 7D respectively, where the peak emission can be seen to be reduced by more than 50% due to the presence of phenol red. It is clear from these results that phenol red reduces the fluorescence emission of different fluorophores excited at 370 nm. This suggests that phenol red acts as a quencher due to a combination of the facts that phenol red is a potent absorber of radiation below 600 nm (figure 6), and yet is minimally fluorescent (figure 7C). When a solution is irradiated, the ability of that solution to absorb incident radiation is dependent on the abilities of the absorbing species present in the solution to absorb at a given wavelength. Thus, it is reasonable to assume that the emission spectrum of a given solution is dependent on the absorbing potential of the different fluorophores present in the solution. Compared to phenol red free DMEM-F12, riboflavin in PBS, and PBS alone (Maguire et al. 2010), the absorption potential of phenol red in PBS at 370 nm far exceeds that of the aforementioned solutions (figure 6). Hence inclusion of phenol red in DMEM-F12 medium, PBS and riboflavin in PBS reduces the intensity of their respective emission spectra since the number of incident photons available for absorption to initiate fluorescence is much reduced compared to the availability in the absence of phenol red.

Furthermore, since phenol red is minimally fluorescent, its presence in the emission spectrum of a solution can be poorly represented despite its ability to modify the optical properties of the solution. This is demonstrated by the visibility of riboflavin in the emission spectrum of DMEM-F12 in the presence (figure 7A) and absence (figure 7F) of phenol red. The peak emission of unirradiated riboflavin occurs at 515-520 nm as shown in both figures 7D and 7E where the presence of phenol red reduces the fluorescence intensity of this peak emission by approximately 50% (figure 7E). Thus, only in the absence of phenol red can the presence of riboflavin be detected in the emission spectrum of unirradiated DMEM-F12, peaking at 515 nm and over shadowing the emission of 10% FBS at 508 nm as shown in figure 7F. Moreover, irradiating riboflavin in the absence or presence of phenol red can be seen to reduce emissions at 515-520 nm and introduce a new emission peak at 460 nm. This new feature at 460 nm is attributed to radiation induced structural alterations of the riboflavin molecule leading to a blue shift (i.e. spectral movement toward shorter wavelengths) in the absorption and emission spectra of the modified riboflavin molecule, thus reducing emissions at 515-520 nm. However, it can be seen that the emission at 515-520 nm is still the dominant emission in the presence of phenol red (figure 7E). This is attributed to the ability of phenol red to absorb incident radiation more effectively than riboflavin resulting in fewer alterations of riboflavin molecules and thus less emission at 460 nm compared to the emission spectrum of irradiated riboflavin in the absence of phenol red.

Based on the fluorescence results, the effects of phenol red and riboflavin on cell survival were investigated. All medium changes were carried out immediately post exposure for all variant PBS exposures to limit the length of time cells were without nutrients. To minimise differences between direct DMEM-F12 and L-15 exposures, cells irradiated in L-15 cell culture medium would ideally be maintained in L-15 for the full 7 days prior to clonogenic assay with no medium change post exposure. However, HaCaT cells cultured in L-15 showed reduced proliferative abilities compared to cells cultured in DMEM-F12, which concurs with the results of Werner et al. (2005) who observed decreased proliferative rates in HepG2 cells cultured in riboflavin deficient medium, thus eliminating the possibility of L-15 clonogenic expansion. Hence, it was necessary to find a time point to perform medium changes at which changing the medium of direct DMEM-F12 exposures would yield survival not significantly different from DMEM-F12 exposures with no medium change. This medium change time point was found to occur 24 hours post exposure (figure 8) which was deemed unsuitable for L-15 exposures due to the reduced proliferative capacity of HaCaT cells in L-15. Medium changes performed 1 to 8 hours post exposure yielded survival not significantly different from one another but all significantly increased with respect to no medium change (figure 8). Since there was no difference between changing the medium 1 to 8 hours post irradiation, a median time point of 4 hours was employed for cells exposed in L-15 before the stored DMEM-F12 was re-introduced.

The survival curves for cells irradiated in the aforementioned solutions are shown in figure 9. All media resulted in reduced but not significantly different survival following a 10 minute exposure (figure 9) which concurs with the survival of cells irradiated in DMEM-F12 medium for 10 minutes and receiving a medium change immediately post

exposure (figure 8). A 60 minute exposure resulted in little or no survival irrespective of the exposure medium. However, a 30 minute exposure resulted in mean survival rates of 37% for PBS, 23% for L-15, 17% for phenol red, 14% for phenol red and riboflavin together and less then 1% survival for cells irradiated in riboflavin. The results demonstrate that riboflavin in PBS is the most phototoxic solution which suggests that riboflavin is the primary component responsible for the dramatic dose response observed in direct DMEM-F12 medium exposures. In agreement with the fluorescence results, the survival curves shown in figure 9, demonstrate that the presence of phenol red with riboflavin during irradiation, lessens the cell killing effect of riboflavin.

Discussion

Previously, it was observed that the survival of HaCaT cells irradiated in DMEM-F12 medium was significantly reduced compared to identical exposures in PBS, despite PBS possessing superior optical transmittance properties that ensures cells irradiated in PBS would receive a larger dose than their counterparts irradiated in DMEM-F12 medium (Maguire et al. 2010). Interestingly, this effect was observed when cells were irradiated with two different solar simulators but not a UVB fluorescent lamp which suggests this effect is wavelength dependent. Based on these intriguing observations, the augmented cell killing abilities of DMEM-F12 medium was further investigated in the present study.

The potential role of ROS in DMEM-F12 medium mediated cell death was investigated using antioxidants. Both GSH and catalase, which are potent inhibitors of hydrogen peroxide, were found to increase survival significantly, with catalase being significantly more effective than GSH. This was surmised to be due to differences in cellular uptake since cells can readily uptake GSH but not catalase (Dahle et al. 2005), which in turn suggests that hydrogen peroxide is produced predominantly in the extracellular medium. This was confirmed when cell death incurred in unexposed recipient cells treated with irradiated blank DMEM-F12 medium immediately post exposure was diminished by both GSH and catalase but GSH was found to be more effective than catalase and GSH supplemented direct exposures. The increased efficacy of GSH is attributed to the absence of cells and thus no reduction in the extracellular concentration of GSH present during blank DMEM-F12 irradiation. These results provide strong evidence that solar simulated radiation induced cell death in DMEM-F12 medium is mediated by the extracellular generation of hydrogen peroxide.

Direct exposures were found to incur increased cell killing compared to indirect exposures. This may be the results of (i) the time taken to exact immediate medium transfers thus treating recipient cells with DMEM-F12 with reduced levels of ROS compared to cells directly exposed, (ii) direct interaction of incident radiation with cells during exposure and / or (iii) factors secreted by irradiated cells. This latter possibility was investigated by irradiating cells of varying density in DMEM-F12 medium, harvesting the medium immediately post exposure and transferring to unirradiated recipient cells. A clear and significant reduction in recipient cell survival was observed with increasing donor cell density. This donor cell density dependent reduction in survival may be the result of cell mediated increases in extracellular ROS. When examining the effect of hydrogen peroxide supplementation on intracellular ROS, Garg and Chang (2004) found that fluorescence of the oxidised derivative of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), 2',7'-dichlorofluorescein diacetate (DCF-DA), was predominantly extracellular. Due to the fact that hydrogen peroxide could not oxidise H_2DCF-DA in the absence of cells, Garg and Chang (2004) concluded that hydrogen peroxide permeablised the plasma membrane thereby facilitating the efflux of intracellular ROS into the extracellular environment. Thus, it is probable that radiation induced extracellular generation of hydrogen peroxide in DMEM-F12 medium promotes increased intracellular ROS that diffuse out into the extracellular environment due to hydrogen peroxide mediated plasma membrane permeablisation. In addition to the

582 extended lifespan of hydrogen peroxide with respect to other ROS such as the hydroxyl radical and superoxide (Bergendi et al. 1999), the high reactivity of the ROS like the
584 hydroxyl radical causes it to exert its effects in the vicinity of their formation (Valko et al. 2007). Thus, if irradiated donor cells do 'leak' ROS into their extracellular environment,
586 it is highly probable that the species involved is hydrogen peroxide.

588 While both the direct exposures and the medium transfers performed immediately post exposure can be adequately explained in terms of ROS generation, the medium transfer
590 experiments performed 1, 24 and 48 hours post exposure are not as straightforward. In ionising radiation studies, cell secreted factors can increase cell killing in an effect termed
592 the bystander effect which has been demonstrated to be donor cell density dependent using medium transfer techniques (Mothersill and Seymour 1997, and has also been
594 reported in response to UV radiation (Banerjee et al. 2005, Whiteside and McMillan 2009). Irradiated blank non-supplemented DMEM-F12 medium transfers at 1, 24 and 48
596 hours post exposure were performed to serve as controls for donor cell medium transfer experiments since Mothersill and Seymour (1997) found that irradiated blank medium
598 had no effect on recipient cell survival. Thus, cell death incurred when recipient cells were treated with irradiated blank DMEM-F12 medium 1, 24 and 48 hours post exposure
600 (figure 3) was highly unexpected and further confounded when it was found that donor cell medium transfers performed 1, 24 and 48 hours post exposure did not incur cell death
602 (data not shown). In fact, donor cell medium transfers were found to yield survival not significantly different from controls at all the late times of transfer (1, 24 and 48 hours)
604 and irrespective of donor cell density. These results infer that solar simulated radiation degrades the nutrient quality of DMEM-F12 but the presence of cells during irradiation
606 provides a protective effect when the medium is transferred to unirradiated recipient cells at or later than 1 hour post exposure. It has been reported that hydrogen peroxide is stable
608 in cell free solutions (Garg and Chang 2004), in fact it has been reported that hydrogen peroxide produced in cell free solutions in response to UVA irradiation retained its
610 cytotoxicity for at least 40 minutes post irradiation (Sato et al. 1995). Thus, blank DMEM-F12 transfer experiments were repeated in the presence of antioxidants. As
612 mentioned, DMSO and catalase had no effect on survival but similar to the immediate GSH blank medium transfers, blank GSH supplemented DMEM-F12 was also found to
614 result in survival not significantly different to controls at all the late times of transfer (data not shown). Since no protective effect was observed due to the presence of catalase
616 at the late transfer times, this eliminates the possibility that hydrogen peroxide maintains a cytotoxic threshold in blank DMEM-F12 medium for 1 hour or longer. Thus, cell death
618 incurred by irradiated blank DMEM-F12 medium at the late times of transfer (figure 3) must be due to solar simulated radiation induced degradation of DMEM-F12 which GSH
620 has the capacity to overcome. The fluorescence results show that solar simulated irradiation of riboflavin causes blue shifts in the emission spectrum of riboflavin that
622 persists for at least 48 hours. Such spectral movements toward higher energy wavelengths (blue shifts) are indicative of structural changes in the form of conjugation losses
624 (Candeias et al. 2001). Thus, it is surmised that solar simulated radiation degrades and defunctionalises riboflavin in DMEM-F12 in a dose dependent manner. Therefore, it is
626 concluded that the survival of unirradiated recipient cells treated with blank irradiated DMEM-F12 post exposure (figure 3) is dependent on the exposure duration (which

determines the degree of nutrient degradation) of DMEM-F12. These dose response curves are not dependent on the time of transfer post irradiation for two reasons; (1) all unirradiated recipient cells treated with blank irradiated DMEM-F12 were seeded 16 hours prior to treatment and are thus assumed to be identical in terms of density and growth phase at the time of treatment regardless of the time of medium transfer post irradiation and (2) the nutrient content of irradiated DMEM-F12 cannot recover or further degrade post irradiation unless further treatments are administered. The enhanced cell death observed at the immediate transfer time point in figure 3 is attributed to the transient production of ROS in blank DMEM-F12 in addition to the nutrient degradation. This conjecture agrees with observations that cell culture media degrade when exposed to background lighting (Grzelak et al. 2001). Riboflavin is the precursor to coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Campbell 1996, Werner et al. 2005). FAD is involved in various metabolic processes such as protein folding in the endoplasmic reticulum (Papp et al. 2005, Werner et al. 2005) and the redox cycle of glutathione where FAD is a coenzyme for glutathione reductase (Schulz et al. 1982, Becker et al. 1995, Werner et al. 2005). In light of the fluorescence results supporting defunctionalisation of riboflavin, it is believed that GSH supplementation not only serves to increase the capacity of cells to process endogenous ROS by increasing the intracellular ratio of reduced to oxidised glutathione (GSH:GSSG), but also enables coenzymes formed from the remaining functional riboflavin to participate in metabolic processes other than the redox cycle of glutathione such as protein folding. The increased survival of recipient cells treated with DMEM-F12 irradiated in the presence of donor cells is attributed to cell secreted factors. This effect is currently under investigation and may be due to transforming growth factor beta (TGF-β1) secreted into DMEM-F12 medium by the donor cells since similar effects have been reported in α–particle radiation bystander investigations (Lehnert et al. 1997, Narayanan et al. 1997, Iyer et al. 2000).

Furthermore riboflavin has been reported to produce hydrogen peroxide when irradiated (Jernigan 1985, Grzelak et al. 2000). However, absorption spectroscopy clearly demonstrated that DMEM-F12 is optically dominated by the presence of FBS and phenol red while riboflavin, at the concentration employed in DMEM-F12, was found to be minimally absorbing. Despite this, the effect of directly irradiating cells in the presence of riboflavin was investigated. Both individual and composite solutions of riboflavin and phenol red at the same concentrations found in DMEM-F12 medium and riboflavin free cell culture medium L-15 and PBS alone were investigated. The production of ROS in cell culture media when irradiated with visible or UVA radiation has been previously reported to be reduced when supplemented with 10% FBS (Edwards and Silva 2001, Mahns et al. 2003), thus FBS was not investigated for photosensitising effects.

It was found that the presence of phenol red in PBS produced increased cell killing above that observed with PBS alone ($p \leq 0.05$). However, the results also show the presence of phenol red to reduce the cell killing abilities of riboflavin, which in the absence of phenol red results in virtually no survival following a 30 minute exposure. Although exposures performed in phenol red with and without riboflavin produce survival not significantly different from one another ($p=1.00$), exposures with phenol red alone exhibit a slight positive effect in the absence of riboflavin. These results concur with the literature

(Grzelak et al. 2000, 2001) and the fluorescence results by showing phenol red to be a mild photosensitiser that possesses significant quenching abilities.

It is clear from the 30 minute exposures in figure 9 that the most phototoxic solution is riboflavin supplemented PBS which suggests that riboflavin is the primary component responsible for the dramatic dose response observed when cells are irradiated in DMEM-F12 medium. This conjecture is supported by preliminary results (two independent tests with 3 replicates per independent test) for cells irradiated in DMEM-F12 medium for 30 minutes and receiving a medium change immediately post exposure. Cells irradiated for 30 minutes in DMEM-F12 medium resulted in survival rates between 0.5% and 4% (data not shown) which was found to be not significantly different ($p=1.00$) to the survival of cells irradiated for 30 minutes in riboflavin supplemented PBS. The phototoxic abilities of riboflavin are further demonstrated by the absence and presence of riboflavin in cell culture media during irradiation. A 10 minute exposure in L-15 and DMEM-F12 medium resulted in significantly different survival rates ($p\leq0.05$) of approximately 80% and 35% respectively when the exposure medium is replaced 4 hours post exposure. Furthermore, the survival of cells irradiated for 30 minutes in L-15 medium was found to be significantly increased ($p\leq0.05$) with respect to the survival of cell irradiated for 30 minutes in DMEM-F12 medium despite receiving a medium change 4 hours later than the DMEM-F12 exposures. It is possible that further increases in cell survival would be observed if cells irradiated in L-15 medium received medium changes immediately post exposure, since it is unlikely that riboflavin is the only photosensitiser present in cell culture media. This may explain the reduced survival of cells irradiated for 30 minutes in L-15 medium compared to their respective counterparts irradiated in PBS. However, this reduced survival is attributed to the presence of phenol red, since phenol red supplemented PBS exposures produced cell survival similar to cells irradiated in L-15 medium. Additionally, tryptophan and tyrosine among others have been found to be negligibly oxidising when irradiated in the absence of riboflavin (Grzelak et al. 2001).

The results present strong evidence that the photosensitiser in DMEM-F12 medium responsible for the extracellular production of hydrogen peroxide is riboflavin. Photosensitisation by riboflavin has been demonstrated to occur by both type I and type II photosensitising mechanisms (Edwards and Silva 2001). It is known that singlet oxygen, which is formed by type II mechanisms, inactivates the enzymatic activity of catalase (Escobar et al. 1996, Kim et al. 2001). However, the presence of catalase during solar simulated irradiation does not appear to impair its antioxidant properties which suggests that riboflavin photosensitisation in DMEM-F12 medium occurs primarily by electron transfer processes (type I mechanisms) similar to that previously reported (Ito et al. 1993). However, Edwards and Silva (2001) observed reduced catalase activity that saturated at 30% deactivation when catalase was irradiated with visible radiation in the presence of riboflavin. Thus, it is possible that type II mechanisms also occur, but to a lesser extent than type I mechanisms, when DMEM-F12 medium is irradiated with solar simulated radiation. This may serve to explain the difference in efficacy between GSH and catalase induced survival in unexposed recipient cells treated with irradiated blank DMEM-F12 medium supplemented with GSH or catalase. Furthermore, Kim et al (1993) have reported that the type and quantum yield of oxidative species generated by flavin

photosensitisers, such as riboflavin, is dependent on the concentration of electron donors and acceptors in solution with the flavin photosensitiser which may serve to explain the slight, albeit not significant, reduction in cell survival post direct irradiation in the presence of SOD. It is possible that superoxide is generated in DMEM-F12 also via riboflavin photosensitisation that may, as a result of the dismutation process in the presence of SOD, further increase radiation induced extracellular concentrations of hydrogen peroxide. This was not further investigated at present since the effect of SOD was not significant.

It is well documented in the literature that riboflavin is a photosensitiser (Joshi et al. 1987, Kim et al. 1993, Edwards and Silva 2001, Keynes et al. 2003, Besaratinia et al. 2007) that absorbs at 365 nm and 420 nm (Ali and Naseem 2002). In fact, it is for this reason that Mahns et al (2003) recommends using PBS as the exposure medium during UVA irradiation. In non ionising radiation studies, cell culture medium with or without phenol red and PBS appear to be the primary exposure media employed in the literature, with a possible bias toward the use of PBS. However, rarely is it mentioned why a particular media was chosen. Previously we have demonstrated that the response of cells during irradiation is not only dependent on the spectral distribution and irradiance of the irradiator employed but also the exposure medium (Maguire et al. 2010). Although we showed differences in cell survival elicited when cells were irradiated in PBS and DMEM-F12, differences also exist between studies that employ seemingly identical exposure media. For example, Sato et al. (1995) concluded that hydrogen peroxide is the primary source of toxicity in a riboflavin supplemented PBS solution irradiated with UVA radiation. In contrast, Mahns et al. (2003) found that hydrogen peroxide was not generated in a riboflavin supplemented PBS solution unless tryptophan was also present during UVA irradiation. Since the quantum yield for superoxide is high in flavin photosensitisers in the absence of electron donors (Kim et al. 1993), it is probable that the discrepancy lies in the PBS supplementation where Sato et al (1995) employed PBS supplemented with inorganic salts and glucose while Mahns et al. (2003) used non supplemented PBS.

Such discrepancies highlight the need to identify a suitable exposure medium that can be employed in UVA, UVB and solar simulated radiation investigations with minimal modifications, in order to aid not only the biological relevance of an investigation but also inter lab comparison. Although we have demonstrated that PBS possesses superior transmittance properties (Maguire et al. 2010), it is known that the capacity of radiation to penetrate the skin decreases with decreasing wavelength (Freeman et al. 1989, de Gruijl 1997). Using ex vivo full thickness epidermal samples, it has been shown that UV radiation is predominantly absorbed in the epidermal layer of the skin with less than 20 % of the incident radiation at 365 nm exiting the basal layer (Bruls et al. 1984). Thus, PBS does not simulate in vivo conditions by permitting increased transmission of short wavelengths to proliferating cells which are found predominently in the basal layer, which would not occur in vivo due to absorption in suprabasal layers. The biological irrelevance of PBS is further demonstrated by the absence of nutrients in the extracellular environment during irradiation since keratinocytes in the non vascularised epidermal layer obtain nutrients that diffuse across the basal membrane into the extracellular space

(Schallreuter and Wood 1995). Thus under 'normal' conditions, in vivo epidermal keratinocytes are not devoid of nutrients in their extracellular environment during solar irradiation. The importance of this has been demonstrated in vitro (Kuhn et al. 1999) and in vivo (Lewis et al. 2009) where human keratinocytes irradiated with UVB radiation in the absence of exogenous growth factors that activate IGF-IR (insulin like growth factor I receptor) were significantly more apoptotic than those irradiated in the presence of IGF-IR activators. The pro-survival response of IGF-IR activation was found to result in post mitotic cells whereas IGF-IR inactivated cells that survived irradiation were fully capable of proliferating with potentially mutagenic genomes.

Although cell death is a convienient end point to examine, it is important to note that cells may achieve this outcome through very different means. As discussed above, the presence or absence of electron donors during irradiation appears to determine the type of ROS generated when riboflavin is photosensitised. Thus, while no significant difference in cell survival was observed between cells irradiated for 30 minutes in riboflavin supplemented PBS and DMEM-F12 medium (data not shown), the mechanisms by which cell death is achieved may be significantly different. This emphasises the need for both mechanistic and mutagenic investigations. Such studies are currently underway.

Whilst it is recognised that in vitro experimentation will never truly mimic the in vivo situation, it is imperative that in vitro studies attempt to bridge the gap separating them in order for progress to be made in skin carcinogenesis investigations. Thus, based on evidence in the literature and the results presented here, it is recommended that serum supplemented riboflavin free cell culture media is employed as the exposure medium during non-ionising radiation investigations. Since radiation is not only capable of riboflavin photosensitisation but also defunctionalisation, it has the potential to further confound investigations if cells are maintained in the exposed medium for extended durations post irradiation. Furthermore, the ability of phenol red to quench riboflavin photosensitisation suggests that phenol red free cell culture media are substantially more phototoxic not only because of uninhibited photosensitisation of riboflavin but also because amino acids such as tryptophan and tyrosine have been demonstrated to augment the oxidising potential of riboflavin (Grzelak et al. 2001). Thus, it is further recommended that phenol red free cell culture media are not used during non ionising radiation investigations.

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Figure legends

Figure 1 Spectral irradiance of the Q-sun solar simulator at the exposure level (▬▬) measured using a Ocean Optics USB2000 miniature fibre optic spectrometer calibrated using spectroradiometry and typical solar irradiance for Florida at noon midsummer (▬ ▬ ▬) as provided by the manufacturer Q-Panel demonstrating the environmental relevance of the Q-sun solar spectrum

Figure 2 Direct exposure dose response plots for HaCaT cells irradiated in DMEM-F12 with and without the presence of SOD (1 mg/ml), DMSO (0.5% v/v), GSH (5mM) or catalase (20μg/ml). Cells were irradiated using the Q-sun solar simulator for (□) 2 minutes (0.8 Jcm^{-2}), (■) 5 minutes (1.9 Jcm^{-2}), (■) 10 minutes (3.8 Jcm^{-2})or (■) sham irradiated (0 Jcm^{-2}) where solid and thatched bars indicate the absence and presence of antioxidants respectively; data presented as the mean ± standard deviation for n = 3 independent experiments, * indicates significant difference between time matched exposures, $p \leq 0.05$.

Figure 3 Indirect exposure dose response plots; blank (no cells) DMEM-F12 irradiated, harvested, filtered and transferred to unirradiated recipient cells immediately (0hr), 1, 24 and 48 hours post exposure. Recipient cells were treated with blank DMEM-F12 irradiated in the Q-sun solar simulator for (□) 2 minutes (0.8 Jcm^{-2}), (■) 5 minutes (1.9 Jcm^{-2}), (■) 10 minutes (3.8 Jcm^{-2}) or (■) sham irradiated (0 Jcm^{-2}); data presented as the mean ± standard deviation for n = 3 independent experiments, * indicates significant difference between the starred dose and its respective control, $p \leq 0.05$.

Figure 4 Indirect exposure dose response plots with and without antioxidants. Blank (no cells) DMEM-F12 with and without antioxidants (DMSO (0.5 % v/v), GSH (5 mM) or catalase (20 μg/ml)) irradiated, harvested, filtered and transferred to unirradiated recipient cells immediately post exposure. Recipient cells were treated with blank DMEM-F12 irradiated in the Q-sun solar simulator for (□) 2 minutes (0.8 Jcm^{-2}), (■) 5 minutes (1.9 Jcm^{-2}), (■) 10 minutes (3.8 Jcm^{-2}) where solid and thatched bars indicate the absence and presence of antioxidants respectively; data presented as the mean ± standard deviation for n = 3 independent experiments, * indicates significant difference between time matched exposures, $p \leq 0.05$.

Figure 5 Donor cell medium transfer dose response plots. Donor cells seeded at a density of 0, $1x10^5$, $2\ x10^5$, $5\ x10^5$ or $1\ x10^6$ cells per well in 3 ml DMEM-F12 were irradiated for 10 minutes (3.8 Jcm^{-2}, ▨) in the Q-sun solar simulator or sham irradiated (0 Jcm^{-2}, ■). Immediately post exposure the donor cell medium was harvested, filtered and transferred to unirradiated recipient cells. Data presented as the mean ± standard deviation for n = 3 independent experiments; * indicates significant difference between irradiated and sham irradiated controls, all exposures tagged with Ɏ are significantly

different to one another; § indicates significant difference with respect to donor cell density $1x10^5$, $p \leq 0.05$.

Figure 6 Transmittance spectra for DMEM-F12 (1 ▬), undiluted FBS (2 ▪▪▪▪), phenol red in PBS (3 – – – –), phenol red and riboflavin in PBS (4 ▬▪▪▬), Riboflavin in PBS (5 ----), phenol free DMEM-F12 (6 — — —), and phenol free DMEM-F12 with 10 % FBS (7 ▬▪▬) where the troughs indicate absorption maxima.

Figure 7 Emission spectra of irradiated (▪▪▪▪) and unirradiated (▬) DMEM-F12 cell culture medium (A), FBS (B), 0.00863 g/l phenol red in PBS (C), 0.00022 g/l riboflavin in PBS (D), 0.00863 g/l phenol red and 0.00022 g/l riboflavin in PBS (E), phenol free DMEM-F12 with 10 % FBS (F), PBS (G) excited at 370 nm. Irradiated solutions were irradiated for 10 minutes (3.8 Jcm^{-2}) in the Q-sun solar simulator and the emission spectra measured immediately post exposure.

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Figure 8 Survival of HaCaT cells irradiated in the Q-sun for 10 minutes (3.8 Jcm^{-2}) in DMEM-F12 and receiving a medium change post exposure or not (none). Data presented as the mean ± standard deviation for n = 3 independent experiments; * implies significant difference with respect to the 10 minute exposure that did not receive a medium change post exposure (i.e. none), $p \leq 0.05$.

Figure 9 Direct exposure dose response curves for cells irradiated in the Q-sun solar simulator. Cells were irradiated in PBS (O), Phenol red (0.00863 g/l) in PBS (●), Phenol red (0.00863 g/l) and Riboflavin (0.00022 g/l) together in PBS (△), riboflavin (0.00022 g/l) in PBS (▲) and L-15 riboflavin free cell culture medium (□) where a 10, 30 or 60 minute exposure corresponds to a dose of 3.8, 11.4 and 22.9 Jcm^{-2}. Data are presented as the mean ± standard deviation for n = 3 independent experiments.