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Title	Intra- and extracellular reactive oxygen species (ROS) generated by blue light
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Citation	Journal of biomedical materials research Part A, 77A(3), 470-477 https://doi.org/10.1002/jbm.a.30663
Issue Date	2006-02-15
Doc URL	http://hdl.handle.net/2115/14473
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Type	article (author version)
Note	The title when publishing in the magazine [Intra- and extracellular reactive oxygen species generated by blue light]
File Information	5JBMRROS2006.pdf

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Intra- and extra-cellular reactive oxygen species (ROS) generated by blue light

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Running Title: Intra- and extra-cellular blue light-induced ROS generation

JOURNAL: J Biomed Mater Res Part A.

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SYNOPSIS

Blue light from dental photopolymerization devices has significant biological effects on cells. These effects may alter normal cell function of tissues exposed during placement of oral restorations, but, recent data suggest that some light-induced effects also may be therapeutically useful, for example in the treatment of epithelial cancers. Reactive oxygen species (ROS) appear to mediate blue light effects in cells, but the sources of ROS (intra- vs. extra-cellular) and their respective roles in the cellular response to blue light are not known. In the current study, we tested the hypothesis that intra- and extra-cellular sources of blue light-generated ROS synergize to depress mitochondrial function. **Methods:** Normal human epidermal keratinocytes (NHEK) and oral squamous cell carcinoma (OSC2) cells were exposed to blue light (380-500 nm; 5-60 J/cm^2) from a dental photopolymerization source (quartz-tungsten-halogen, 550 mW/cm^2). Light was applied in cell-culture media or balanced salt solutions with or without cells present. Intracellular ROS levels were estimated using the dihydrofluorescein diacetate (DFDA) assay; extracellular ROS levels were estimated using the leucocrystal violet assay. Cell response was estimated using the MTT mitochondrial activity assay. **Results:** Blue light increased intracellular ROS equally in both NHEK and OSC2. Blue light also increased ROS levels in cell-free MEM or salt solutions, and riboflavin supplements increased ROS formation. Extracellularly applied ROS rapidly (50-400 μM, < 1 min) increased intracellular ROS levels, which were higher and longer-lived in NHEK than OSC2. The type of cell-culture medium significantly affected the ability of blue light to suppress cellular mitochondrial activity; the greatest suppression was observed in DMEM-containing or NHEK media. Collectively, the data support our hypothesis that intra- and extracellularly generated ROS synergize to affect cellular mitochondrial suppression of tumor cells in response to blue light. However, the identity of blue light targets that mediate these changes remain unclear. These data support additional investigations into the risks of coincident exposure of tissues to blue light during material polymerization of restorative materials, and possible therapeutic

benefits.

INTRODUCTION

Blue light (wavelengths of 380-500 nm) is commonly used *in situ* to polymerize dental resin restorative composites that contain the photoactivator camphorquinone (Abs$_{max}$ 462 nm). High radiant flux (300-3000 mW/cm^2) of blue light is emitted from these dental photocuring devices (for comparison, room light is about 5-10 mW/cm^2), but the biological effects of high-intensity visible light, and in particular blue light, are not well characterized in most tissues. In dentistry, visible light and blue light are currently considered innocuous to oral tissues and are used without consideration of adverse biological effects. However in the retina, overexposure to high intensity visible or blue light contributes to retinal oxidative stress and possibly the onset of age-related macular degeneration.[1,2]

Accumulating evidence indicates that *visible light* (400-700 nm) is not without biological effects in tissues besides the retina. At high doses visible light may inhibit mitosis, suppress mitochondrial activity, or damage DNA.[3,4] Some evidence indicates that visible light also may have therapeutic benefits.[5-9] The intensities and exposure times used in all these studies have often been poorly defined and are not relevant to dental curing sources, but together, these results support a full accounting of both the biological risks of visible light and possible therapeutic effects.

Relatively recent research indicates that *blue light* from dental photocuring devices also has biological effects. Quartz-tungsten-halogen (QTH), laser, and plasma-arc dental sources were lethal to mouse fibroblasts *in vitro* at levels below 15 J/cm^2, yet these levels did not produce significant (< 1° C) heat generation.[10] Other evidence indicates that blue light may have therapeutic effects, particularly through its ability to inhibit cellular division.[11,12,13] In particular, blue light has been suggested as a potential therapeutic treatment for oral epithelial cancer in *in vitro* models.[14] Animal and human studies also have demonstrated the potential utility of blue light for cancer therapy.[15,16]

The mechanisms by which blue light affects cells remain unclear, but roles for cellular

porphyrins and flavins as 'targets' of blue light have been proposed.[14,17,18,19] Flavins and porphyrins[18,20,21] are among the few cellular molecules known to absorb blue light, and flavins have been implicated as both intracellular and extracellular absorbers of blue light.[17,22,23] Flavins (from riboflavin, vitamin B-2, 7, 8-dimethyl-10 isoalloxazine) occur intracellularly as FMN and FAD, but these species exist only sparsely (40-60 nM) in the serum and extracellular matrix.[24] The role for porphyrins has been largely defined in the context of photodynamic therapy,[21,25] but their high concentrations in the mitochondrial inner membrane makes them suspects as intracellular targets of blue light.[11,13]

Flavins and porphyrins are capable of transducing blue light into reactive oxygen species (ROS).[18,21,25] However, the extent to which this occurs in cells or tissues is not known. The emerging roles of ROS in mediating cellular function and survival[26] make them a reasonable focus as a mediator of blue light-induced cellular effects. ROS have been measured *in vitro* in blue light-exposed cells,[17,27] and intracellular ROS levels and processing appear to correlate with the differential responses of tumor vs. normal cells to blue light *in vitro*.[14,27,28] These data support a hypothesis that blue light induced-ROS mediate, at least in part, the suppression of mitochondrial function. However, the roles of intra- and extracellular sources of the ROS in causing blue light effects, a possible role for riboflavin or flavins *in vitro* in generating ROS, and the degree to which these two sources interact to cause cellular responses are unclear. In the current study, we show that both intra- and extra-cellular ROS are generated by blue light in epithelial cultures, and that both mediate blue light induced-mitochondrial responses. Our data support further study about possible clinical risks during photocuring of restorative materials as well as therapeutic uses for blue light, but suggest that the extracellular matrix is one critical modulator of the blue light response.

MATERIAL AND METHODS

Cells and cell-culture

Normal human epidermal keratinocytes (NHEK) and tumor (oral squamous carcinoma,

OSC2) cells were used based on previous studies that demonstrated that OSC2 cells were less sensitive to the cytotoxic effects of blue light than NHEK.[11,14] These contrasting responses, and the differential levels of ROS induced by blue light in these cell types[27] also provided a good model in which to identify the source of blue light-generated ROS and the contributions of intra- and extra-cellular ROS to cell responses. NHEK (CC2507, Cambrex) were maintained in KGM2 (Cambrex) and used within 2 passages of thawing. OSC2 (provided courtesy of Dr. Tokio Osaki, Japan) were maintained in 50/50 vol% DMEM/F12 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL of hydrocortisone (all from Gibco BRL). For experiments, cells were plated in 96-well flat-bottom plates at 40,000 cells/cm^2 in 200 μL medium and were incubated 24 h before blue light exposure. The cells were exposed to light in Hallam's buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES) or various cell-culture media. Hallam's was used as an extracellular matrix that did not produce ROS in response to blue light. In this manner, the ROS induced intracellular effects of light could be monitored independently of those in cell-culture medium, where externally generated R0S may have contributed to intracellular ROS load and mitochondrial suppression.

Light source and exposure conditions

A quartz-tungsten halogen (QTH) source, filtered to provide blue light (380-500 nm) was used to irradiate the cells (Optilux, Model 501, SDS-Kerr). This device, which is commonly employed in the photopolymerization of dental restorations, generated a radiant flux of 550 mW/cm^2 in the 380-500 nm range and had an output tip with a 10.4 mm diameter that completely covered the culture well (inside diameter 6.5 mm, Fig. 1). The distance from the light tip to the cell monolayer was 9 mm, with 6 mm occupied by the Hallam's. Attenuation of light energy at the level of the monolayer was about 10% (measured in pilot studies and previously reported [27]). Attenuation was neutral to wavelength in the 380-500 nm range.

Light was applied to cultures (n = 3) for continuous periods of 10 s (5 J/cm^2) to 240 s (120

J/cm^2, Fig. 1). Exposure of other cultures in the plate to coincident light was prevented by placing sterile tissue paper into the wells between cultures. Control cultures received no light (see below for details), but were present on the same plate as the light-exposed wells to ensure identical times of exposure to coincident (room) light and incubation times. Temperature increases in the cultures were previously measured and were less than 3°C for less than 3 min.[10] In pilot studies, these temperature increases did not alter cell mitochondrial activity.

Intracellular ROS (DFDA assay)

The DFDA assay was used to estimate intracellular ROS levels. DFDA (dihydrofluorescein diacetate) is taken up by cells, then activated by esterase-mediated cleavage of acetate, which is trapped in the cell. The dihydrofluorescein may then be oxidized by ROS to fluoroscein, which has a measurable fluorescence. Cells were washed with 200 μL/well of Hallam's buffer, then 100 μL/well of Hallam's was re-added to maintain cellular viability. During all measurements, cells were kept in the dark as much as possible. DFDA (5 μM, Molecular Probes[29]) was then added. Previous reports have suggested that light autoconverts the DF probe to fluorescein and DFDA (and resulting intracellular DF) should therefore not be present during irradiation.[30] Thus, we structured our experiments in two ways to assess the impact of this possible artifact. In the first method, DFDA (5 μM) was added in 100 μL of Hallam's before light exposure. Light was applied, then fluorescence (excitation 485 nm, emission 530 nm, 20 nm bandwidths, 37°C) was measured 10 s, 15, 30, 45, 60, and 90 min post-light exposure (Bio-Tek Instruments, Inc.). In the second method, light was applied first, then DFDA was added and fluorescence measured after the DFDA addition. Negative controls, without light, cells, or diamide, were used to establish baseline ROS levels. Positive controls received no light, but 5 mM diamide (Sigma), a known oxidative stressor,[27] was added at the same time as light exposure. For all experiments, fluorescence was expressed as a percentage of the diamide positive controls (with negative controls subtracted) to standardize oxidative responses between experiments.

Extracellular ROS (LCV assay)

Generation of light-induced hydrogen peroxide (H_2O_2) in the various cell-culture media was assessed using the leucocrystal violet (LCV) assay.[31] LCV is converted to a chromophore absorbing light at 596 nm upon oxidation with peroxide. Samples of Hallam's, MEM or DMEM without phenol red, or these solutions supplemented with 0.5 or 4.0 mg/L riboflavin (common cell-culture medium concentrations, Sigma) were tested with or without blue light irradiation. Medium containing serum or phenol red was not tested because pilot studies established that phenol red and serum quenched the LCV. H_2O_2 standards were prepared (0-500 μM, in equivalent media) as positive controls. Samples or standard solutions (20 μL) were added to 96-well flat-bottom wells with 80 μL of acetate buffer (sodium acetate, 2 M, pH 4.5), 70 μL H_2O, 10 μL horseradish peroxidase (HRP, 5 mg/mL), and 20 μL LCV solution (0.5 mg/mL in 0.5% HCl, Sigma 219215). After complete mixing, color indicating the presence of H_2O_2 was read immediately at 596 nm.

Succinate dehydrogenase (SDH) activity

The succinate dehydrogenase (SDH) activity of NHEK or OSC2 cells was measured using the MTT method.[31] Cells were exposed to light in either a 50/50 vol% mixture of OSC2 and NHEK medium, DMEM with 10% FBS, or MEM and 10% FBS in 96-well culture wells (n = 3) as described above, then incubated for 72 h before assessing SDH activity. Controls received no-light, and SDH activity was expressed as a percentage of these controls. In equivalent cultures, 200 μM of H_2O_2 was added immediately after light treatment. Pilot experiments established that 200 μM did not significantly suppress SDH activity of either NHEK or OSC2 cells.

RESULTS

Intracellular ROS generation from blue light

Adding DFDA to Hallam's prior to blue light exposure caused a significant increase in intracellular ROS levels over those when DFDA was added immediately after light application (Fig. 2). The increased levels observed when DFDA was added before irradiation were suspect as artifacts due to light-induced fluorescein conversion based on previous literature (see introduction). Thus, in all other experiments where light was applied, DFDA was added as soon as possible (2-5 s) after light exposure.

Blue light induced detectable intracellular ROS levels in a dose-dependent fashion from 5-60 J/cm^2 when light was applied in Hallam's solution (Fig. 3). Levels were higher, but not statistically higher, in NHEK than in OSC2 at all light energies. At 120 J/cm^2, intracellular ROS levels were markedly lower (by 70% vs. 60 J/cm^2) in both cell types, presumably due to cytotoxicity.

ROS generation in media or Hallam's

Addition of H_2O_2 (2-500 μM) to Hallam's and MEM (without phenol red) induced the predicted increase in LCV activation (Fig. 4). This level of peroxide was nontoxic to both NHEK and OSC2 cells after 72 h exposure (data not shown). Blue light induced ROS in the MEM and Hallam's in a dose-dependent manner, with 120 J/cm^2 inducing about 1/20th of levels induced by 250 μM of H_2O_2. ROS induced in Hallam's was higher than in MEM, but these differences were not statistically significant. The addition of riboflavin (0.5 or 4.0 mg/L) to Hallam's or MEM increased blue light-induced ROS (Fig. 4) above those exposed to light alone (60 J/cm^2) by 50-100%. However, levels of riboflavin/light-induced ROS, even with 4.0 mg/L riboflavin and 60 J/cm^2 of blue light, were about 3% of those induced by 250 μM of peroxide.

External peroxide vs. intracellular ROS.

H_2O_2 added extracellularly quickly (within 1 min) elevated intracellular ROS levels in both

NHEK and OSC2 (Fig. 5). In NHEK, levels were higher. For example, 200-400 μM of extracellularly applied H_2O_2 increased intracellular ROS to 15-20% of diamide-induced levels in NHEK, but only 8-10% in OSC2. In both cell types, intracellular ROS levels dropped over 30-60 min, but reached baseline levels more quickly in OSC2 than in NHEK. For example, 200 μM of H_2O_2 elevated intracellular ROS by 3% in OSC2 and intracellular levels dropped to baseline within 15 min. However the same concentration of H_2O_2 elevated intracellular ROS to 17% of diamide, and levels had not reached baseline (6%) by 60 min.

SDH activity

In NHEK, light doses of 5-60 J/cm^2 alone caused 10-15% decreases in SDH activity (Fig. 6, statistically significant at 30 and 60 J/cm^2). When H_2O_2 was added immediately after irradiation with blue light, SDH activity was suppressed an additional 5-10%, but these decreases were not statistically different from conditions with light alone. The addition of H_2O_2 alone (200 μM) decreased SDH activity about 15% (not statistically significant).

In OSC2, H_2O_2 (200 μM) alone did not significantly alter SDH activity. Blue light alone cause the expected decrease in SDH activity and peroxide addition after irradiation significantly suppressed SDH activity over that of light alone. When these experiments were repeated in DMEM (containing 4 mg/L riboflavin), H_2O_2 (200 μM) had no effect but light alone as well as light-peroxide combinations were uniformly suppressive (>70% vs. negative controls). In MEM (containing 0.5 mg/L riboflavin), H_2O_2 (200 μM) alone was not suppressive and light alone suppressed SDH activity by only 10-15%. However, the combination of H_2O_2 and light was significantly more suppressive, causing 20-60% additional suppression of SDH activity over that of light alone.

DISCUSSION

Controversy persists about ROS artifacts produced by blue-light via intracellular conversion of dihydrofluorescein to fluorescein in the absence of ROS.[30, 32] The current results support the

existence of this artifact (Fig. 2) and suggest that when assessing the effects of blue light with DFDA, DFDA must be added post-exposure to light. Cell-free DFDA controls were used, but could not account for the activation by cells of DFDA via intracellular removal of acetate. Thus, the strategy of post-light DFDA addition was used. The employed strategy carried its own risks, however, because most ROS are very short-lived (nanoseconds,[33]) and even the most conscientious and swift addition of DFDA post-light exposure is bound to be added too late to detect the presence of some ROS. Therefore, the addition post-light exposure almost certainly underestimated ROS formed in response to blue light. In the current study, we chose the 'after' strategy as the most reasonable option.

The current results clearly demonstrate that blue light induces intracellular ROS formation in a dose-dependent manner (Fig. 3). The existence of light-induced intracellular ROS was unequivocal, even accommodating for the DFDA artifacts mentioned in the previous paragraph and accounting for corrections of ROS from the external medium. Doses of 120 J/cm^2 appeared to be cytotoxic in both cell types. However, unlike a previous study,[27] blue light-induced intracellular ROS generation was not statistically different between OSC2 and NHEK cells. Previous data indicated that blue light-induced ROS levels were significantly higher in OSC2 cells than NHEK. These apparently incongruent results likely stem from the sequence and timing of applying the light and the DFDA. In previous experiments, light was applied in native media for each cell type. The cells were then washed and DFDA-Hallam's applied as soon as possible. Thus, differences in the OSC2 and NHEK media (including differences in flavin contents) may have contributed to apparent differences in the intracellular ROS levels, considering the current data (Figs. 4,5).

Several reports have suggested a role for riboflavin in cell-culture medium in influencing intracellular ROS and cellular responses to blue light. [15,19,23] This hypothesis predicts that riboflavin (or other extracellular components) produce ROS that migrate intracellularly and affect cell responses. The current results demonstrate that peroxide concentrations generated in

MEM or Hallam's in response to blue light were relatively low (Fig. 4) compared to levels that affected cellular SDH activity (Fig. 5). Furthermore, the addition of riboflavin to MEM or Hallam's increased blue light-generated ROS significantly, but these increases were very small relative to SDH-relevant peroxide levels. Thus, the current results do not support a role for extracellular riboflavin as a major mediator of blue light-induced SDH suppression. However, the current results do not rule out other flavins such as FAD or FMN, and do not rule out porphyrins, all of which have been suggested as blue light targets.[7,23,34] The markedly different cellular responses to blue light in different media support a role for extracellular medium components in blue light cellular responses (Fig. 6).

Our results (Fig. 6) suggest that both intracellular and extracellular factors are necessary for cellular responses such as SDH suppression to occur in response to blue light irradiation. OSC2 cells exposed to 200 μM peroxide alone responded with far less SDH suppression than when light and peroxide were added, even at sublethal light doses. The results in Fig. 2 show that light alone induces intracellular ROS levels, but Fig. 6 shows that these levels are insufficient by themselves to suppress SDH activity. Fig. 5 supports the rapid movement of peroxide into the cell. Thus, the current results suggest that unknown components in the medium generate ROS in response to blue light which then synergize with intracellularly generated ROS to lead to SDH suppression. The data in Fig. 6 support a hypothesis that media (DMEM, MEM, NHEK) are quite different in their ability to generate extracellular ROS. The extent to which these media components are relevant *in vivo* will play a role in the translation of these *in vitro* experiments to *in vivo* therapies. The low concentrations of free riboflavin in the serum, for example, in combination with the data in Fig. 4, do not support riboflavin as a therapeutically useful blue light target.

In summary, the current results show that blue light generates intracellular ROS independent of a contribution of ROS from the media. However, mitochondrial suppression induced by blue light in OSC2 tumor cells is apparently dependent on both extra- and intra-

cellular contributions of ROS. Components of the medium play a role in the extracellular contribution, but the identity of these components is unclear, except that riboflavin is not a major ROS contributor in this context. These results also support the possibility that blue light poses some risk to tissues exposed coincidentally during the photopolymerization of restorative materials.

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Figure Legends

Fig. 1. Diagram of blue light irradiation geometries. The light source tip (10.4 mm in diameter) was placed onto the 96-well place, which contained 200 μL of Hallam's or medium. Coincident light exposure to adjacent wells was prevented by filling these wells with sterile tissue paper.
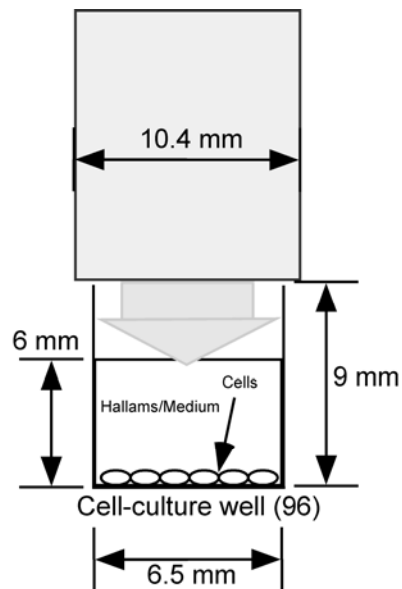
Fig. 2. Intracellular reactive oxygen species (ROS) generated after exposure to blue light (30 J/cm^2 over 60 s) in oral squamous carcinoma (OSC2) cells in Hallam's solution. Dihydrofluorescein diacetate (DFDA) was added to capture ROS in two ways, either before irradiation with the light or as soon after light exposure as possible (generally 5-10 s). Diamide (5 mM) was added at the time of light exposure in control wells. ROS were expressed as a percentage of diamide positive controls, subtracting negative controls that received DFDA but no diamide or light. Error bars indicate standard deviations (n=3). Asterisk indicates statistical differences between the before and after conditions (t-test, two sided, α=0.05).
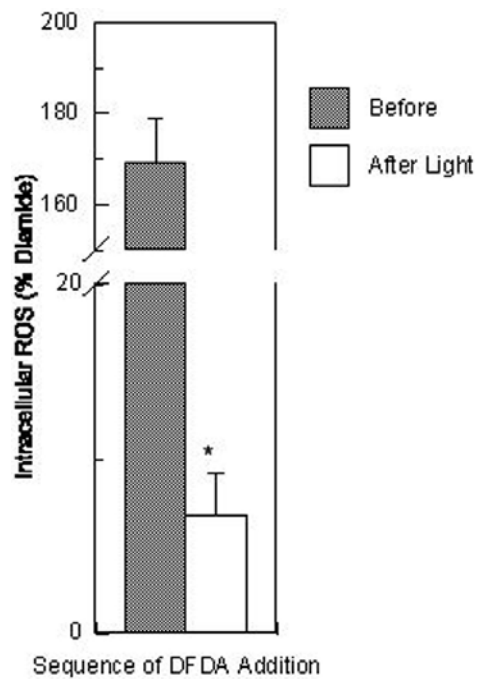
Fig. 3. Intracellular reactive oxygen species (ROS) generated in normal human epidermal keratinocytes (NHEK) or OSC2 cells 2 h after blue light exposure to varying doses (in J/cm^2). ROS were measured in Hallam's to avoid external medium derived I light-induced ROS, using DFDA (added 5-10 s after light exposure) and were expressed as a percentage of 5 mM diamide controls. Although NHEK average ROS levels were higher than OSC2, there were no statistical differences at any dose (t-tests, two sided, α=0.05, n=3).
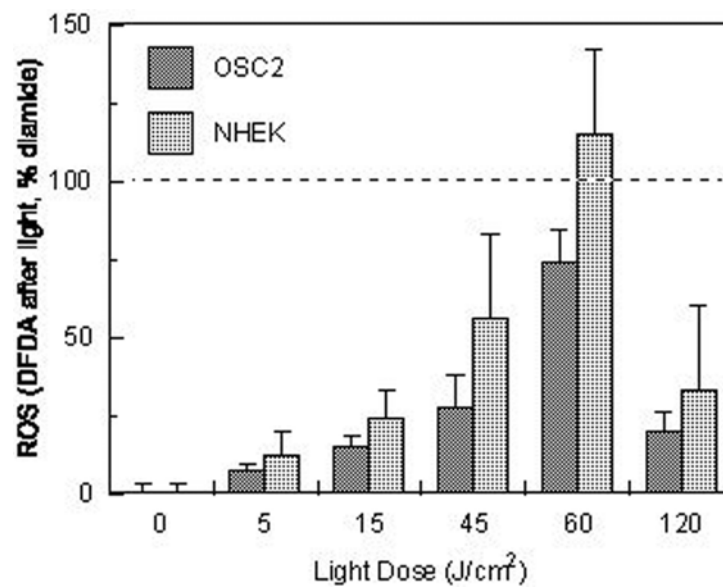
Fig. 4. ROS generated in Hallam's (phosphate-buffered saline with glucose) or MEM (minimum essential Eagle's medium, phenol red-free) medium, assessed by leucocrystal violet (LCV) assay 2 h after light exposure of 5-120 J/cm^2 and expressed as optical density (OD). Hydrogen peroxide (H_2O_2) was added as a positive control at a dose that was sublethal to the NHEK or OSC2 cells. *At top*, ROS generation in medium or Hallam's without supplement. *Middle*, cumulative ROS generation in MEM 2 h after 60 J/cm^2 light exposure, with 0, 0.5, 4 mg/L of riboflavin (a known blue light absorber). Control conditions had no light, no LCV, and no riboflavin added. *At bottom*, the riboflavin experiments were repeated with Hallam's buffer. Asterisks indicate statistical differences between light conditions with or without riboflavin supplementation (t-tests, two sided, $\alpha=0.05$, n=3).
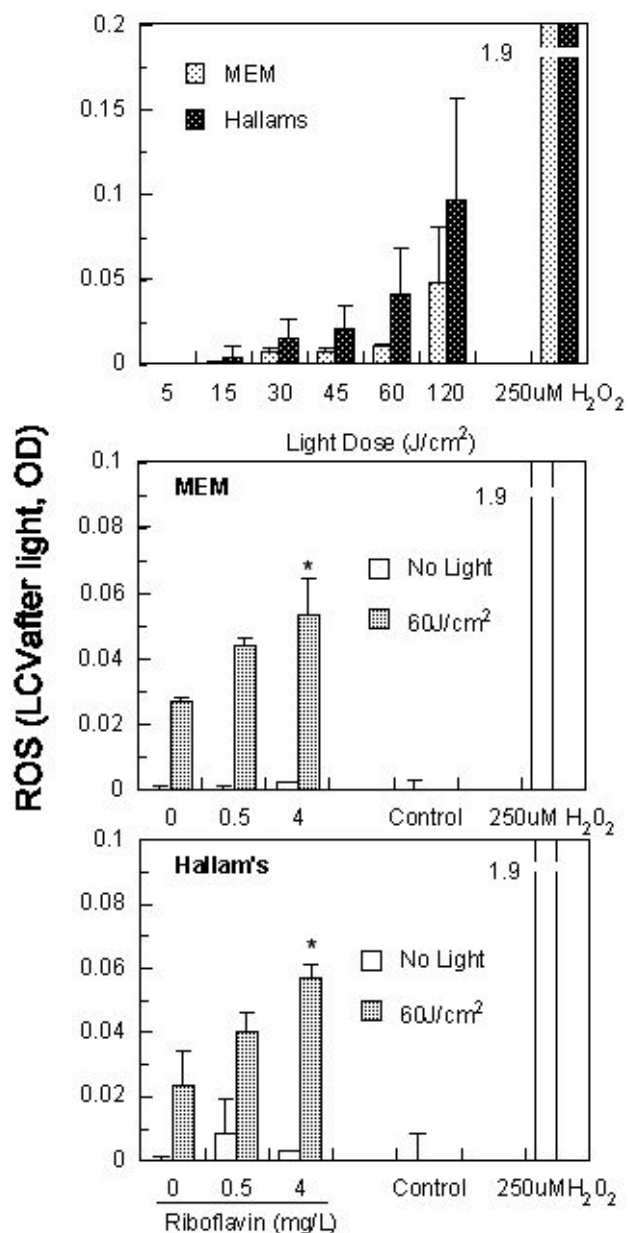
Fig. 5. Intracellular ROS in NHEK or OSC2 cells 0-60 min after addition of hydrogen peroxide

(H_2O_2, 0-400 μM) to cell-culture medium. ROS were measured by DFDA method and expressed

as a percentage of diamide (5 mM) controls. Error bars indicate standard deviations of the mean

(n=3). Note that concentrations of 50 and 100 μM are not shown for OSC2 because they

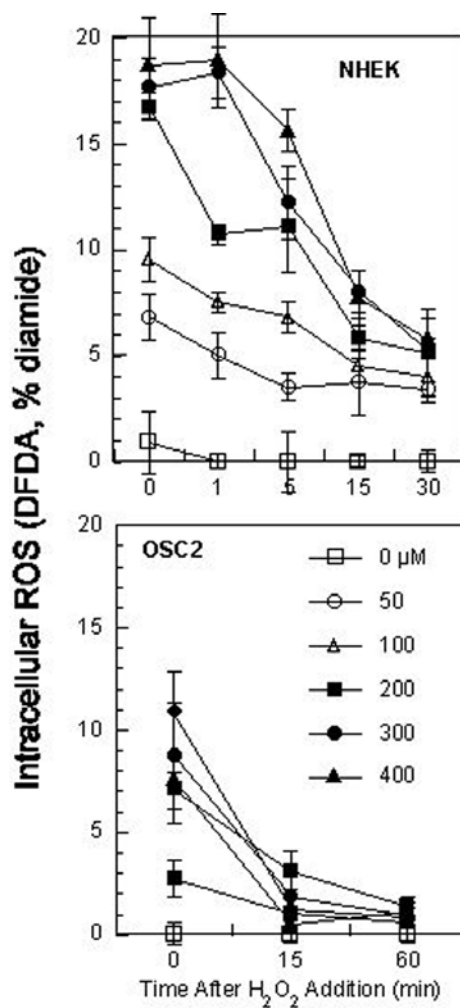caused no detectable increases in intracellular ROS in these cells.

Fig. 6. Activity of succinate dehydrogenase (SDH) in NHEK or OSC2 cells (n =3) 72 h post-exposure to blue light, expressed as a percentage of no-light controls. OSC2 were cultured in either DMEM or MEM with 10% FBS or in a 50/50 vol% mixture of NHEK medium and normal OSC2 medium (see methods). Light was applied in cell-culture media. Error bars indicate standard deviations of the mean (n = 3); asterisks indicate significant differences between H_2O_2-treated and non-treated (2-sided t tests, α = 0.05). Dashed line indicates control SDH activity of no-light, no-peroxide group.