Is the Photosuturing Agent, Rose Bengal, a Mutagen?

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Received December 11th, 2013; revised January 9th, 2014; accepted January 17th, 2014

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ABSTRACT

Rose Bengal (RB) is a potential photosuturing agent that may improve standard dermatologic surgical closure techniques. However, RB produces reactive oxygen species with photoactivation and its photomutagenic potential must be considered in clinical application. We investigated cytotoxicity, mutagenicity, and singlet oxygen (SO) production of RB on epithelial Chinese hamster ovary cell line. Cells were exposed to RB concentrations: 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001%; irradiated for 400 s using a high-intensity visible wavelength lamp or maintained in the dark. Cell viability was assessed by XTT assay, mutagenicity by HPRT gene mutation assay, and SO production by Sensor Green reagent. RB > 0.001% was significantly cytotoxic. Viabilities were uninfluenced by ≤0.0001% RB controls, or 30-min incubation. 49% of irradiated cells died after 24-h in 0.0001% RB. At ≥0.001% RB, >90% of cells died. Irradiating 0.00001% - 0.001% RB increased SO; levels dropped significantly between 0.01% - 0.1%. Controls exhibited negligible SO production. HPRT suggested that RB was not mutagenic (0.0001%, 0.00001%); SO induction increased between 0.00001% - 0.001%, with reduced production at higher concentrations. Pilot studies suggested irradiated 0.0001% RB is mutagenic in vitro; current data suggest RB is not photomutagenic. The contribution of RB’s cytotoxicity on observed clinical improvement of scars and mutagenic potential remains unclear, necessitating further study.

KEYWORDS

Rose Bengal; Photochemical Tissue Bonding; Mutagenicity; Cytotoxicity; Wound Healing; Dermatological Surgery

1. Introduction

Wound healing and scar formation are researched extensively, and especially important to dermatological surgery. Skin incisions and excisions are closed by a variety of suture types, staples, wound closure strips, and adhesives. However, recent research shows promise in improved epidermal wound closure and healing with a photochemical tissue bonding (PTB) agent, 0.1% Rose Bengal (RB:4,5,6,7-tetrachloro-2',4',5',7'-tetraiodo-fluorescein) suspended in phosphate buffered saline (PBS), when applied to wound edges and activated by green laser light [1,2]. Upon photoactivation, Rose Bengal may behave as a sort of “nanosuture,” as the dye may crosslink proteins between tissues surfaces thereby sealing skin wounds [2]. Rose Bengal does not appear to be phototoxic in porcine skin ex vivo or rabbit skin in vivo [3]. However, previous studies have demonstrated that photoactivated RB generates reactive oxygen species (ROS), including singlet oxygen (¹O₂), which promote lipid peroxidation, DNA damage, cell membrane damage, and can cause cell death [4,5]. In this study, we investigated toxicity, mutagenicity, and singlet oxygen production of photoactivated Rose Bengal using epithelial cells in culture.

Rose Bengal, a fluorescein derivative, is a historical Ophthalmologic stain used to aid in diagnosing ocular surface disorders. RB absorbs and is excited by green wavelengths (~532 nm) leading to a tissue bonding reaction when applied to wound edges via an unknown mechanism. Photoactivating RB may aid in wound closure, improve wound healing, promote skin graft adhesion, and cell viability appears unaffected [1,2,6].
many potential benefits of PTB, including simplicity, rapidity, and painlessness. Further, PTB could avoid suture removal, foreign body reactions, additional tissue injury, and infection. The first in-human clinical trial of RB-induced PTB was recently completed in 2009. Results showed that PTB provided adequate tensile strength preventing wound dehiscence and less inflammation compared to sutures, and may also reduce scarring [7].

Research shows photoactivated RB’s ability to cross-link collagen scaffolds and found this is effective for producing strong, stable collagen microstructures with improved physiochemical scaffold properties [8]. PTB is hypothesized to work via a light-initiated covalent cross-linking of proteins between the wound surfaces, leading to the immediate formation of a relatively impermeable seal [7,9,10]. When excited states of the activated photosensitizer and photoproducts (e.g., ROS) are formed, they interact with proximal biomolecules (e.g., amino acids in collagen) leading to their photoexcitation. Photoexcited biomolecules can then react with adjacent side chains forming covalent protein-protein crosslinks [11-13]. Photoactivated RB reaction has shown to favor the production of 1O2 (~75%), the remainder being primarily super activated RB reaction has shown to favor the production of 1O2 (~75%), the remainder being primarily super.

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As a photosensitizer molecule in an aerobic environment, RB’s activation by either ultraviolet or visible light irradiation leads to its excited singlet state (Figure 1) [12,15]. Each photosensitizer (PS) molecule has the potential to generate 10³ - 10⁵ 1O2 molecules prior to reduction with 1O2 or by another reaction, thus exhausting the cycle [12]. With this capacity to regenerate until reacting with proximal biomolecules or auto-exhaustion, there is ample potential for activated photosensitizers to create harmful ROS.

In patients treated with intralesional RB chemo-ablation for subcutaneous melanoma metastases, systemic phototoxicity has been observed [16]. Using RB on HaCat keratinocytes, the production of 1O2 has been found using a spectrophotometer in photosensitized cells. Again, RB’s potential for mutagenicity was not assessed [17]. Because RB can enter living cells and stain the nucleus, this raises questions about mutagenicity, especially since studies have demonstrated that byproducts of Rose Bengal are capable of interacting with and damaging DNA [18,19]. This study examines the cytotoxicity and mutagenicity associated with photoactivated RB in hopes of gaining a better insight into the potential hazards associated with the use of RB in PTB procedures.

2. Materials and Methods

Chinese hamster ovary cells (CHO), a mammalian epithelial cell line with fibroblast-like and epithelial-like morphology, were obtained from American Type Cell Culture (Manassas, Virginia). Fetal bovine serum (FBS), Ham’s F-12, L-glutamine, penicillin/streptomycin, 0.05% trypsin-EDTA solution, and Singlet Oxygen Sensor Green were purchased from Invitrogen (Frederick, MD). 6-thioguanine (6-TG) and 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) were purchase from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) and methanol were purchased from Fisher Scientific (Houston, TX). Crystal violet was purchased from Acros Organics (Geel, Belgium). The high-intensity visible light (81,500 lux; Mastech Digital 4-Range 200,000 Lux Meter, Model: LX1330B) with a 65 Watt fluorescent bulb was purchased from a hardware store.

2.1. Cell Culture and Treatment

CHO cells were cultured in Ham’s F-12 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin in 25 cm² culture flasks in a dark, humidified incubator at 37°C with 5% CO₂. Cells were maintained under exponential growth conditions and RB suspended in Ham’s F-12 was added to control and RB flasks at the time of light irradiation, creating 0%, 0.1%, 0.01%, 0.001%, 0.0001%, or 0.00001% RB concentrations. All exposure groups were maintained in duplicate cultures and handled in dark lighting, avoiding RB photo-activation.

2.2. Light Irradiation

Cells were suspended in Ham’s F-12 with RB and irradiated for 400 seconds to high-intensity visible light (~400 - 700 nm), using a 65 Watt fluorescent bulb (T-max 33.9°C at 400 seconds) by placing culture flasks on glass 3 cm above the bulb [15]. Controls remained in the dark.

2.3. Cell Viability

Trypan Blue staining was used for initial cell counts to assist in calculating appropriate cell quantities necessary for each of the assays used. Cell viability was assessed using the XTT assay (TOX2) according to the manufacturer’s protocol (Sigma-Aldrich), to assess mitochondrial dehydrogenase activity. After 30 minutes or 24 hours post light irradiation, 20 μL of activated XTT solution was added to the cells, then incubated for 3 hours. Six replicates for each exposure concentration were examined. Background absorption was eliminated by centrifuging plates at 200× g for 5 minutes and supernatant was transferred to a new plate. Absorbance at 450 nm

Figure 1. Photosensitization Cycle. PS = Photosensitizer; 1PS* = PS excited singlet state; 3PS* = PS excited triplet state; hv = energy of a photon of UV or visible radiation [12].
(corrected at 690 nm) was measured using a microplate spectrophotometer (Bio-RAD Benchmark Plus).

2.4. HPRT Gene Mutation Assay

The HPRT mutation assay and methods for calculating plating efficiency and mutation frequencies followed Gupta and Singh’s technique [20]. The assay was repeated for each test condition 3 separate times, using 3 plates to determine plating efficiency and 6 plates for 6TG resistance. Cells were maintained in the dark incubator for 5 days to select for mutant colonies, replacing complete medium once on day 3 of incubation. On day 5, medium was removed, cells were rinsed with PBS, stained with Cresyl violet, and colonies were counted.

2.5. Singlet Oxygen Production

Test concentrations of RB (0%, 0.1%, 0.01%, 0.001%, 0.0001%, or 0.00001%) were suspended in Ham’s F-12 or PBS. 10 μL of 5 mM Sensor Green (SG) reagent was added to 90 μL of the range of RB solutions, and 100 μL of RB-SG solution were pipetted into 96-well tissue culture plates, 3 replicates for each concentration with and without light irradiation. Controls were covered with aluminum foil during and after light irradiation, avoiding irradiation. The F-12/SG and PBS/SG plates were irradiated for 0, 200, or 400 seconds. Specimens were evaluated for ROS using a fluorescence microplate reader (Fluostar Optima, BMG Labtech) at the following settings: Excitation 485 nm, Emission 520 nm, Position 3, “Fluorescence.”

2.6. Data Analysis

Dose-response relationships for the examined endpoints were statistically tested using a general linear model (univariate analysis of variance procedure) in SPSS version 16.0 for Windows. Post-hoc mean comparisons were conducted using a Bonferroni correction for multiple comparisons. P-value <0.05 was used to determine significance.

3. Results

3.1. XTT Assay

Viability was unaffected at 0.0001% RB in Ham’s F-12 with and without light irradiation after 30 minutes of incubation (Figure 2). Nearly 45% of cells were lost after light irradiation and 24 hours of incubation with 0.0001% RB (Figure 3). Over 90% of cells died when exposed to 0.001% up to 0.1% RB in Ham’s F-12 regardless of light irradiation, after 30 minutes and 24 hours of incubation. There appeared to be significant dose-dependent negative effects (p < 0.01) in viabilities.

3.1.1. No Light, 30 Minutes Incubation

There was no difference between the control and the lowest dose (0.0001% RB); however, comparing these to the higher concentrations in this group, controls and 0.0001% RB were significantly different from all other concentrations (p < 0.01), with viabilities greatly reduced in the latter concentrations. There were no significant differences amongst other concentrations.

3.1.2. Light, 30 Minutes Incubation

Similar findings occurred within this group as in the “No Light” group above. There was no difference between the control and the lowest dose (0.0001% RB). Comparing these concentrations to higher concentrations in this group, controls and 0.0001% RB were significantly different from all other concentrations (p < 0.01), with greatly reduced viabilities in the latter. There were no significant differences amongst other concentrations.

3.1.3. No Light, 1440 Minutes (24 Hours) of Incubation

Similar findings occurred here as in the “No Light” and
“Light, 30 Minutes of Incubation” groups in Figure 2. There was no difference between controls and lowest dose (0.0001% RB), however, comparing these to the remaining concentrations in this group, controls and 0.0001% RB were significantly different from all other concentrations (p < 0.01) with greatly reduced viabilities in the latter concentrations. There were no significant differences amongst other concentrations.

3.1.4. Light, 1440 Minutes (24 Hours) of Incubation Controls were significantly different from all other concentrations (p < 0.01), with viabilities showing a slight increase after irradiation with light. Additionally, the lowest dose (0.0001% RB) differed from all other concentrations (p < 0.01), where the irradiated group had nearly 50% reduction in viability compared to the group without irradiation. There was no difference amongst all other concentrations.

3.2. HPRT Gene Mutation Assay
The initial HPRT gene mutation assay revealed that irradiating cells with 0.0001% RB suspended in Ham’s F-12 was likely mutagenic, with roughly a doubling of the mutant frequency above background (Figure 4). A repeat study including 0.00001% RB did not appear to be mutagenic at 0%, 0.00001%, or 0.0001% RB in Ham’s F-12 regardless of irradiation status (Figure 5). Higher concentrations were cytotoxic and the assay was not performed.

3.3. Singlet Oxygen Production
The Sensor Green Assay detected $^1$O$_2$ production by RB in PBS (Figure 6) or Ham’s F-12 (Figure 7) at varying concentrations with and without light irradiation for 0, 200, or 400 seconds.

3.3.1. Sensor Green Assay in PBS Buffer
1) PBS, No Light Controls, 0.00001%, 0.0001%, or 0.001% RB did not differ from one another, but were found to be significantly higher than the two highest concentrations, 0.01 and 0.1% RB (p < 0.01). 0.1% RB was significantly different from all other concentrations (p < 0.02), with nearly undetectable levels of $^1$O$_2$ species.

2) PBS, 200 Seconds of Light Controls had significantly lower amounts of $^1$O$_2$ production when compared all other concentrations (p < 0.01), except for the highest concentration, 0.1% RB. Singlet oxygen production for controls and 0.1% RB remained quite low, whereas from 0% to 0.001% RB the levels of $^1$O$_2$ increased. Levels of $^1$O$_2$ production were greatly reduced from 0.001% to 0.01% RB.
3) PBS, 400 Seconds of Light

Controls had significantly lower amounts of $^{1}$O$_2$ production from all other concentrations ($p < 0.01$), except for the two highest concentrations, 0.01% and 0.1% RB. Levels of $^{1}$O$_2$ production increased from 0% to 0.001% RB, but there was a decrease in $^{1}$O$_2$ production between 0.001% and 0.1% RB.

3.3.2. Sensor Green Assay in Ham’s F-12 Medium

1) F-12, No Light

Similar findings as in the PBS, “No Light” group above were discovered. 0.1% RB concentration was significantly different from all groups ($p < 0.02$), with nearly undetectable levels of $^{1}$O$_2$ molecules.

2) F-12, 200 Seconds of Light

Controls produced low levels of $^{1}$O$_2$ species, and were not significantly different from 0.00001% or 0.1% RB. Most of the remaining concentrations significantly differed from one another ($p < 0.01$), with increased $^{1}$O$_2$ production from 0% to 0.001% RB, and decreased detectable $^{1}$O$_2$ from 0.001% to 0.1% RB.

3) F-12, 400 Seconds of Light

There was an increase in $^{1}$O$_2$ production from 0% to 0.001% RB, and reduced detection of $^{1}$O$_2$ levels from 0.001% to 0.1% RB. Controls produced low amounts of $^{1}$O$_2$ and were not significantly different from 0.1% RB, but were significantly different from all other concentrations ($p < 0.01$). All other concentrations were significantly different from one another ($p < 0.01$).

4. Discussion

Finding improved wound closure techniques with few side effects is an important topic [22-24]. RB has only recently been investigated as a PTB agent [1,2,7]. It was initially thought to be harmless and unable to penetrate vital cellular organelles, however, studies show RB stains nuclei and other essential organelles, i.e., in human intestine smooth muscle cells (HISM) [18]. Additionally, photoactivated RB generates ROS and has been used to induce strokes in mice [17,25]. ROS are important for many normal physiological processes, but high levels cause DNA and cell membrane damage, which can be cytotoxic. Studies have investigated RB’s ROS induction, cytotoxicity, staining of cellular organelles, and histological and macroscopic outcomes of PTB, but few address the issue of photoactivated RB-induced mutagenicity [3]. Both UVA and chemical- or photochemically-induced formation of $^{1}$O$_2$ exhibit the ability to modulate gene expression, enzyme induction (i.e., collagenases, and intercellular adhesion molecule-1) [26]. Chemical- or photochemically-induced formation of $^{1}$O$_2$ appears to have effects similar to those of UVA radiation, including activation of c-Jun amino-terminal kinases (JNK) in human skin fibroblasts [15]. $^{1}$O$_2$ has been implicated in altered dermal states arising from abnormal collagen cross-linking from $^{1}$O$_2$ targeting histidine and histidinohydroxysinonorleucine via photooxidation in bovine skin which may reduce tensile properties [27].

Reducing incubation from 5 minutes to seconds, there is rapid dye uptake by stained cell membranes and nucleus in HISM cells with 0.1% RB. Cells incubated for longer periods of time have reduced intracellular fluorescence emission, likely a result of the dye’s self-quenching effect. Regarding RB’s intrinsic toxicity, morphologic changes were exhibited in four cell types (rabbit tendon fibroblasts, cells from bovine pulmonary artery, HISM, and rabbit corneal epithelial cells) in the presence of varying concentrations in the dark and ambient lighting, where higher (1%) concentrations of RB appear to cause cell detachment and loss, and lower (e.g., 0.01%) concentrations reduce cellular motility [18].

With the above information in mind, this study was designed to test the hypothesis that photoactivated RB induces ROS (namely $^{1}$O$_2$) at low concentrations reduces cell viability and increases genotoxicity in epithelial cells. The range of RB concentrations selected increased from 0 to 0.1% RB (concentration recently investigated in human subjects) [1,2,7]. Light irradiation exposure times included 0, 200, and 400 seconds (double the length of time in recent research) to compensate for the lack of a source of concentrated green wavelength. Having piloted a study where cultures exposed to 0% and 0.1% RB in PBS, PBS was itself cytotoxic within 24 hours. As a result, we suspended RB in Ham’s F-12, as this was not overtly detrimental to viability. Green light minimally penetrates the epidermis in animal models, which should hereby localize photodynamic therapies to the epidermis [28]. We irradiated cells with a high intensity light source, containing green wavelengths, and is also minimally penetrating [15].

RB concentrations >0.001% in Ham’s F-12 were very cytotoxic. The XTT assay revealed that viabilities were uninfluenced by low RB concentrations (i.e., ≤0.0001% RB) maintained in the dark, or after 30 min of incubation. These values are quite low compared to concentrations used in recent human research (i.e., 0.1% RB) [27]. Perhaps at RB concentrations as low as 0.0001%, there is not enough $^{1}$O$_2$ production to exhaust the cells’ innate ability to overcome the detrimental effects of ROS at these low concentrations. Time appears to play a role in the effect on viability, with 49% of irradiated cells being lost after 24 hours of incubation in concentrations as low as 0.0001% RB. This is possibly due to an increased amount of time the cells were exposed to oxidative stress and the associated detrimental effects. At ≥0.001% RB, the effects were even more obvious with >90% of cells being lost. This supports a dose-dependent negative effect on viability and a possible interaction with light and time at the lowest concentration out to 24 hours. Again,
the resulting cytotoxicity from these low RB concentrations (e.g., 0.001%) is well below the 0.1% concentration used in clinical studies.

The Sensor Green reagent revealed that irradiating 0.00001% - 0.001% RB increased amounts of detectable $^1\text{O}_2$ species, with a significant reduction between 0.01% - 0.1%. While an increase in $^1\text{O}_2$ production with increasing RB concentrations seems relatively intuitive, the data concerning 0.01% - 0.1% RB concentrations were unexpected. Perhaps this observation can be explained by photoactivated RB’s ability to undergo auto-exhaustion during shorter periods compared to lower concentrations. Assessing $^1\text{O}_2$ production at varying time points might be a way to investigate these observations. Alternatively, perhaps higher concentrations of RB absorb or mask Sensor Green’s fluorescence as these concentrations are much more heavily pigmented than lower concentrations. As expected, controls exhibited almost no $^1\text{O}_2$ production.

The initial HPRT gene mutation assay showed that irradiating cells with 0.0001% RB in Ham’s F-12 was possibly mutagenic, with roughly a doubling of the mutant frequency above background. However, results from the second experiment using lower RB concentrations suggested that RB was not mutagenic at 0.001% or 0.00001% concentrations. This is not unreasonable since the highest concentration is quite low (~1 μM). All other concentrations resulted in low or nonexistent colony formation due to RB’s cytotoxicity. Qualitatively, when comparing the HPRT groups to controls (colony forming plates), which were elevated beyond expected, there were no apparent differences in frequency of HPRT mutations amongst experimental groups. In our opinion, the assay did not meet the quality standards that we set, and several experiments could be repeated. Investigating the mutagenicity of photoactivated RB on skin cell lines should also be considered.

5. Conclusion

With the current information at hand, it appears that higher concentrations of RB on viable cells are relatively cytotoxic. Singlet oxygen production increased from 0 to 0.001% RB, with detectable levels decreasing from 0.001% to 0.1% RB for unknown reasons. Further research might include a shorter time period between photoactivation and microplate fluorometry to rule out the auto-exhaustion theory, as well as different time points between exposure and ROS measurement. Given that photoactivated RB is currently being tested as a PTB agent on open wounds, additional research on the potential mutagenicity of RB needs to be done. Should RB prove not to be mutagenic, future research might focus on determining the exact mechanisms and/or effects of PTB on wound healing and scar formation. Perhaps PTB has potential to reduce infection, additional post-operative pain, and poor cosmetic outcomes. Of note, photoactivated RB has recently shown promise as a fungicidal agent against *Trichophyton rubrum* [29]. Other items that should be considered include the potential for an increased risk of future complications, such as localized pigment disorders or even skin malignancies.

REFERENCES


