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Assessing Daylight & Low-Dose Rate Photodynamic Therapy Efficacy, Using Biomarkers of Photophysical, Biochemical and Biological Damage Metrics in Situ.

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ABSTRACT

Background: Sunlight can activate photodynamic therapy (PDT), and this is a proven strategy to reduce pain caused byconventional PDT treatment, but assessment of this and other alternative low dose rate light sources, and their efficacy, has not been studied in an objective, controlled pre-clinical setting. This study used three objective assays to assess the efficacy of different PDT treatment regimens, using PpIX fluorescence as a photophysical measure, STAT3 cross-linking as a photochemical measure, and keratinocyte damage as a photobiological measure.

Methods: Nude mouse skin was used along with in vivo measures of photosensitizer fluorescence, keratinocyte nucleus damage from pathology, and STAT3 cross-linking from Western blot analysis. Light sources compared included a low fluence rate red LED panel, compact fluorescent bulbs, halogen bulbs and direct sunlight, as compared to traditional PDT delivery with conventional and fractionated high fluence rate red LED light delivery.

Results: Of the three biomarkers, two had strong correlation to the PpIX-weighted light dose, which is calculated as the product of the treatment light dose (J/cm^2) and the normalized PpIX absorption spectra. Comparison of STAT3 cross-linking to PpIX-weighted light dose had an R = 0.74, and comparison of keratinocyte nuclear damage R = 0.70. There was little correlation to PpIX fluorescence. These assays indicate most of the low fluence rate treatment modalities were as effective as conventional PDT, while fractionated PDT showed the most damage.

Conclusions: Daylight or artificial light PDT provides an alternative schedule for delivery of drug-light treatment, and this pre-clinical assay demonstrated that in vivo assays of damage could be used to objectively predict a clinical outcome in this altered delivery process.

1. Introduction

Conventional photodynamic therapy (PDT) using 5-aminolaevulinic acid (ALA) is commonly used to treat actinic keratosis (AK) [1,2], with some investigational and some approved uses in squamous cell carcinoma (SCC) [3], and basal cell carcinoma (BCC) [4–6]. Despite ALA-PDT being highly effective to treat AKs and non-melanoma skin cancers, patients often report moderate to severe pain associated to the treatment [7–10], and this has been viewed as one of the more problematic issues in acceptance of the treatment. The source of pain in ALA-PDT is believed to be from protoporphyrin IX (PpIX) production or accumulation in nerve endings [11] which leads to damage during illumination. This pain has been related to the PpIX concentration in AK lesions [12].

To date, several studies have reported daylight-mediated PDT as effective as conventional PDT to treat AK lesion grade I with reduced

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pain [13]. Since "daylight PDT" consists in a low rate PDT light delivered by the sun for extended periods of time with either none or low incubation time of the photosensitizer, then the PpIX is produced at the same time that it is photobleached away in the treatment process [14–17]. So, the delivery process of daylight PDT appears effective with sunlight, however at the same time this process of no incubation time with continuous irradiation could also be easily achieved with lamps in a clinical setting, where the light delivery and patient behavior might be better controlled. The development of this paradigm with low pain but effective light delivery in a clinical setting could be a successful conduit for increased use of PDT, if demonstrated to be equally effective as traditional PDT delivery.

In the present study, we investigated the hypothesis that "daylight PDT" using different light sources could be as effective as conventional PDT, using the well-established model of normal nude mouse skin [18,19]. The study used three in vivo biomarkers of PDT treatment efficacy, including PpIX fluorescence assessed by fiberoptic dosimetry [15,20–22], damage to epidermal keratinocytes assessed by pathology [23], and induction of STAT3 cross-linking [24–26] as assessed by molecular analysis of biopsy samples, and examined these in response to different light sources as well as conventional PDT and fractionated PDT [27].

2. Materials and methods

2.1. PDT treatment

All animal studies were approved by Dartmouth College Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with institutional PHS and OLAW guidelines.

Seventy female nude mice were used (Charles River Laboratories, Wilmington, MA) and separated into 10 animals/group with groups: (1) untreated control (no ALA, no light), (2) sunlight, (3) halogen, (4) Compact Fluorescent Light (CFL), (5) red LED, (6) traditional PDT, and (7) fractionated light PDT (fPDT). Groups 5–7 used the same red (633 nm) LED panel (Omnilux revive, Sydney, Australia), but group 5 was set at a lower fluence rate. All groups were prescribed a total light dose of ~78 J/cm^2, except for the sun (107 J/cm^2), which could not be easily controlled. The "effective" light dose was calculated by using a PpIX-weighted irradiance [28,29] (details in supplementary data).

A volume of 20 μL Levulan® Kerastick® (20% of ALA) solution was applied topically to the back of each mouse. In the "daylight" groups (sun, halogen, CFL, and LED), the animals received light immediately after ALA application for 2.5 h. In the single-illumination group (PDT), the animals were illuminated after 2.5 h of ALA application. In the fPDT group, the animals were illuminated twice, first after 1.25 h of dark incubation, and then again at 2.5 h after the initial ALA application (Fig. 1). The ALA was not reapplied during nor between any illuminations. During "daylight" treatment, the animals were awake for the duration of the procedure, while during PDT and fPDT, the mice were

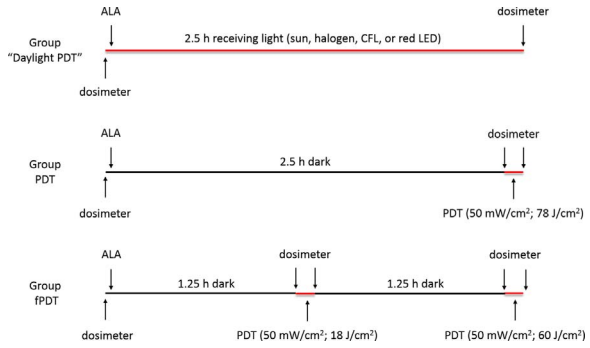

Fig. 1. Schematic of the different PDT regimens is shown with light delivery shown by the red lines, relative to the time of application of ALA to the skin (left side).

anaesthetized with isoflurane.

Light treatments were given for the different light sources, directly measuring the optical irradiance and time of light delivered to the surface of the tissue. The spectrum of each source relative to the absorption spectrum of PpIX was used to calculate the PpIX-weighted irradiance, which was the product of the normalized PpIX absorption spectrum and the treatment light as multiplied wavelength by wavelength and then integrated together [28,29]. This process does not take into account individual variation in PpIX production, but rather just estimates an 'effective' irradiance related to the light source, which allows comparison between light sources from the theoretical efficiency of how they should excite PpIX. Details of the measurement systems and exact calculations are in Supplementary data. Time integrated irradiance was then reported as the delivered light dose.

2.2. Fluorescence dosimetry measurements

Active dosimetry of PpIX was done using optical measurements of remitted fluorescent intensity were acquired with both 405 nm laser (blue channel) and 635 nm laser (red channel) excitation. These measurements were corrected with white light reflectance measurements, to correct for attenuation due to individual variation. All optical measurements were collected using a previously reported point-probe dosimetry system and the analysis was carried out by applying an iterative Monte Carlo-based look-up-table (LUT) fitting algorithm [22]. For "daylight PDT" groups, the measurements were done (1) prior ALA administration (*Pre-ALA*) and (2) after light illumination (*Post-PDT*). For the regular PDT group (PDT), measurements were performed at three time points: (1) prior to ALA (*Pre-ALA*), (2) immediately before PDT (*Pre-PDT*) (data not showed), and (3) after treatment (*Post-PDT*). For the fractionated light illumination group (fPDT), the measurements were performed at five time points: (1) prior to ALA application (*Pre-ALA*), (2) before 1st light fraction (*Pre-1st PDT*), (3) after 1st light fraction (*Post-1st PDT*), (4) before 2nd light fraction (*Pre-2nd PDT*), and (5) after 2nd light fraction (*Post-2nd PDT*). All measurements were acquired with the probe gently in contact with the back of the mice, where the ALA was applied. The post-PDT PpIX fluorescence (*PpIX FL_{norm}*) was normalized by subtracting the average skin auto-fluorescence obtained before ALA application ($\overline{FL}_{Pre-ALA}$) from the average fluorescence obtained post-PDT (or post-2nd PDT, for fPDT group) ($\overline{FL}_{Post-PDT}$) for each mouse and for both the blue and red excitation channels.

$$PpIX\ FL_{norm}\ (a.u.) = \overline{FL}_{Post-PDT} - \overline{FL}_{Pre-ALA} \quad (1)$$

2.3. Western blot analysis

The proteins from skin were extracted immediately after light treatment using RIPA buffer containing protease and phosphatase inhibitors and 1 mM of PMSF. The skin was kept cooled by ice for approximately 20 min before electric homogenization, followed by centrifuge for 5 min at 13,000 rpm. The supernatant was transferred to a clean tube, and this sample volume was frozen at −20 °C. This was repeated for 5 mice/group.

Protein extracts (50 μg) were briefly heated at 100 °C in βME-containing buffer, separated on a polyacrylamide gel (4–15% Criterion™ TGX™, Bio-Rad), and transferred to 0.2 μm PVDF membrane (Trans-Blot® Turbo™ Mini PVDF Transfer, Bio-Rad). On all gels, reference protein markers for molecular size detection (Precision Plus Protein Standards Kaleidoscope, Bio-Rad, #161-0375) were included. Non-specific interactions were blocked by incubating the membranes with 0.1% Tween 20, 5% powder milk in PBS for 1 h at room temperature. Membranes reacted overnight at 4 °C with primary antibodies (anti-STAT3 C-20, Santa Cruz, 1:500; anti-β-actin N-21, Santa Cruz, 1:500). Detection of the immune complexes were performed using a fluorescent secondary antibody, 1:15,000 (IRDye® 800CW Goat anti-Rabbit IgG, LI-

COR Biosciences). Membranes were then scanned on the Odyssey CLx Infrared System (LI-COR Biosciences) in 800 nm channel. The quantification of each band was determined using the Image Studio Lite Software (LI-COR Biosciences). The relative amount of STAT3 cross-linking was expressed by the percentage conversion of monomeric STAT3 into the dimer form I [25]:

$$STAT3\ cross-links\ (\%\ form\ I) = \frac{form\ I}{monomeric\ STAT3} \times 100 \quad (2)$$

2.4. H & E staining and microscope analysis

At 24 h post treatment, the skin was excised, samples fixed overnight in 4% neutral buffered formaldehyde, and then routinely processed and embedded in paraffin for sectioning into 4 μm thickness and H & E staining. These sections were scanned on a 20 × objective microscope, and 2 sections per slide were chosen at random to be digitally scanned for analysis. PDT induced damage was assayed manually by visually counting nuclei. The majority of the morphological abnormalities was observed in the epidermis, so for simplicity and consistency the dermis was excluded. Other exclusion criteria were cells surrounding hair follicles and melanocytes because of their irregular frequency of appearance in the scanned sections.

To quantify damage, all keratinocytes appearing in the epidermis were counted and classified as either normal or aberrant. Nuclei were considered aberrant if they appeared pyknotic (dark and condensed chromatin), hyper eosinophilic (increased pink cytoplasm), shrunken, fragmented, or washed out. To normalize, the number of aberrant cells was simply rationed over the total number of cells counted per section.

2.5. Statistical analysis

Statistical analysis was performed with the normality of the distribution evaluated according to the Shapiro-Wilk's test. For the analysis of difference between normal distributions, a parametric Student's *t*-test was performed. A non-parametric Mann-Whitney was used to compare groups with non-normal distributions. Linear regression was used to test correlation between STAT3 cross-linking, damage to epidermal keratinocytes, PpIX fluorescence, and the PpIX-weighted spectrum; it was reported as Pearson product correlation coefficient (R). *P*-value < 0.05 was considered statistically significant. Variables shown in figures with different overhead letters represent statistically significant differences. All statistical analyses were performed in OriginPro® 8 (OriginLab, Wellesley Hills, MA, USA), with the exception of the linear regression tests which were done in Python (Version 3.4.3, SciPy package version 0.19.0).

3. Results

3.1. Total dose vs. PpIX-weighted, "effective" dose

While care was taken to match the total light dose (J/cm^2) between groups, the PpIX-weighted light dose, calculated as the product of the normalized PpIX absorption and the time-integrated treatment light spectra, presented significant variability due to differences in excitation spectra (Table 1).

3.2. in vivo measurement of PpIX fluorescence

To evaluate if there was photoactivation and destruction of the photosensitizer after "daylight PDT", the post-PDT PpIX fluorescence (*PpIX* FL_{norm}) in response to either blue or red excitation channel was determined and the results compared with "conventional" (single scheme or fractionated) PDT. Fig. 2 shows that all treatments were effective in promoting no accumulation of PpIX, with each scheme of treatment used varying slightly in effect. There are some statistical

Table 1
A comparison of the unweighted and PpIX-weighted irradiance and dose for each light source.

Light Source	Measured Wavelength Range (nm)	Irradiance (mW/cm^2)	Total light Dose (J/cm^2)	PpIX-weighted Irradiance (mW/cm^2)	PpIX-weighted light Dose (J/cm^2)	$FL_{Pre\text{-}ALA}$ (blue channel) (mean ± SD)	$FL_{Post\text{-}PDT}$ (blue channel) (mean ± SD)	$FL_{Pre\text{-}ALA}$ (red channel) (mean ± SD)	$FL_{Post\text{-}PDT}$ (red channel) (mean ± SD)
Sun	350–800	11.9	107	0.46	4.1	0.0033 ± 0.00076	0.041 ± 0.021	0.0083 ± 0.0042	0.10 ± 0.061
Halogen	350–800	8.6	77	0.20	1.8	0.0040 ± 0.0013	0.11 ± 0.-074	0.0078 ± 0.0073	0.26 ± 0.20
CFL	350–800	8.6	77	0.47	4.2	0.0022 ± 0.00056	0.045 ± 0.019	0.0054 ± 0.0031	0.15 ± 0.078
Red LED	633	8.6	77	0.16	1.4	0.0036 ± 0.0017	0.081 ± 0.027	0.0087 ± 0.0035	0.16 ± 0.055
PDT	633	50	78	0.92	8.3	0.0042 ± 0.0017	0.067 ± 0.048	0.010 ± 0.0039	0.11 ± 0.061
fPDT	633	50	18, 60	0.92	1.9, 6.4	0.0036 ± 0.0011	0.028 ± 0.017	0.0088 ± 0.0036	0.046 ± 0.035

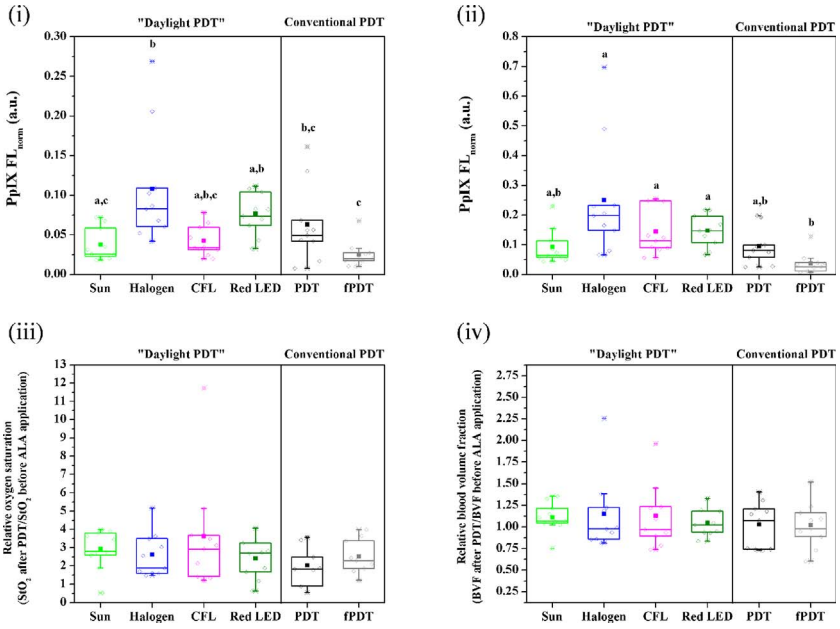

Fig. 2. Normalized post-PDT PpIX fluorescence, *PpIX FL$_{norm}$*, measured in response to both blue (i) and red (ii) excitation channel, showing high PpIX activated photobleaching in all treatment groups. Relative oxygen saturation (iii) and relative BVF (iv) estimates were determined by white light spectroscopy of the skin with the dosimeter probe. Letters shared indicate no significant difference.

differences between individual treatment groups, but overall daylight PDT and conventional PDT were similar in their capacity to photoactivate PpIX.

As expected, there was a replenishment of PpIX during the dark interval between 1st and 2nd scheme of illumination from fPDT group (data shown in Fig. S4). No significant difference was observed for either relative oxygen saturation or relative blood volume fractionation (BVF) between treatment groups (Fig. 2).

3.3. STAT3 protein cross-linking assay

The influence of either daylight PDT or conventional PDT (PDT and fPDT) on STAT3 cross-linking was evaluated by measuring the percentage of STAT3 monomer conversion into form I (Fig. 3). The data showed that all regimens of PDT induced cross-linking of STAT3 relative to the control group (P-value < 0.05); however, there was no significant difference between "daylight PDT" and conventional PDT (P-value > 0.05). The results also show that the conversion of STAT3 monomer in form I was highest in mice that received fPDT.

3.4. Evaluation of damage to keratinocytes caused by different treatment regimens

Fig. 4 presents the damage to epidermal keratinocytes as a result of "daylight PDT" or conventional PDT. Its quantification showed a more pronounced damage in upper epidermis caused by fPDT. Among "daylight PDT" groups, sun and CFL were the treatments that caused the most damage, followed by halogen. There was no statistical difference when comparing sunlight, halogen, or CFL with PDT. Almost normal appearing, with occasional classic (apoptotic) sunburnt cells were seen for low fluence rate red LED.

3.5. Biometric assays correlation with PpIX-weighted, effective light dose

In order to evaluate the efficacy of the bioassays previously described, we compared the PpIX-weighted light dose of each treatment group to the quantitative assessment of each assay (Fig. 5). There was little correlation of PpIX fluorescence to the effective light dose ($R = -0.46$ [red ex.], -0.37 [blue ex.], data not shown), but significant linear correlations to both STAT3 cross-linking ($R = 0.74$) and keratinocyte damage ($R = 0.70$).

4. Discussion

In this study, the potential implementations of low dose rate or "daylight PDT" were further investigated as compared to traditional PDT delivery or fractionated PDT. The "daylight PDT" is here defined as low-irradiance PDT light dose delivered by the sun, or artificial lamps, immediately after application of ALA to the skin [30,31]. This has been reported in many studies to be a low-pain methodology of delivery [32], which could lead to wider adoption of PDT as a self-delivered treatment. This activity produced the motivation for the central hypothesis of this study, which was that the use of different low-irradiance light sources could produce skin damage equally as efficiently as

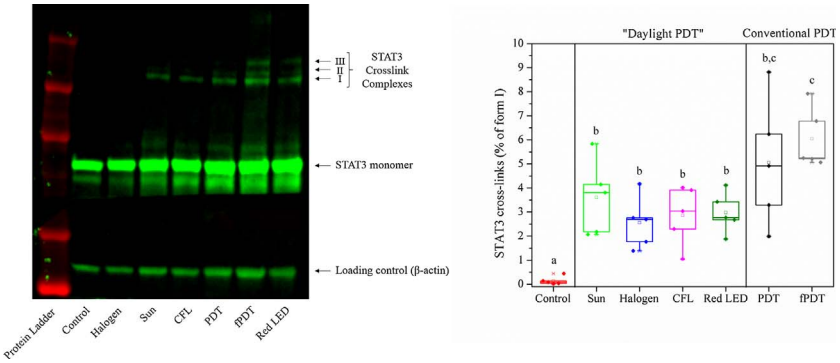

Fig. 3. Left: Representative Western blot showing STAT3 cross-linking after ALA-PDT treatment. Right: Quantitative effect of "daylight PDT" and conventional PDT on STAT3 cross-linking (% of monomer conversion into form I). The values for individual mice were plotted ($n = 5$ mice/group). Distinct letters mean statistical difference.

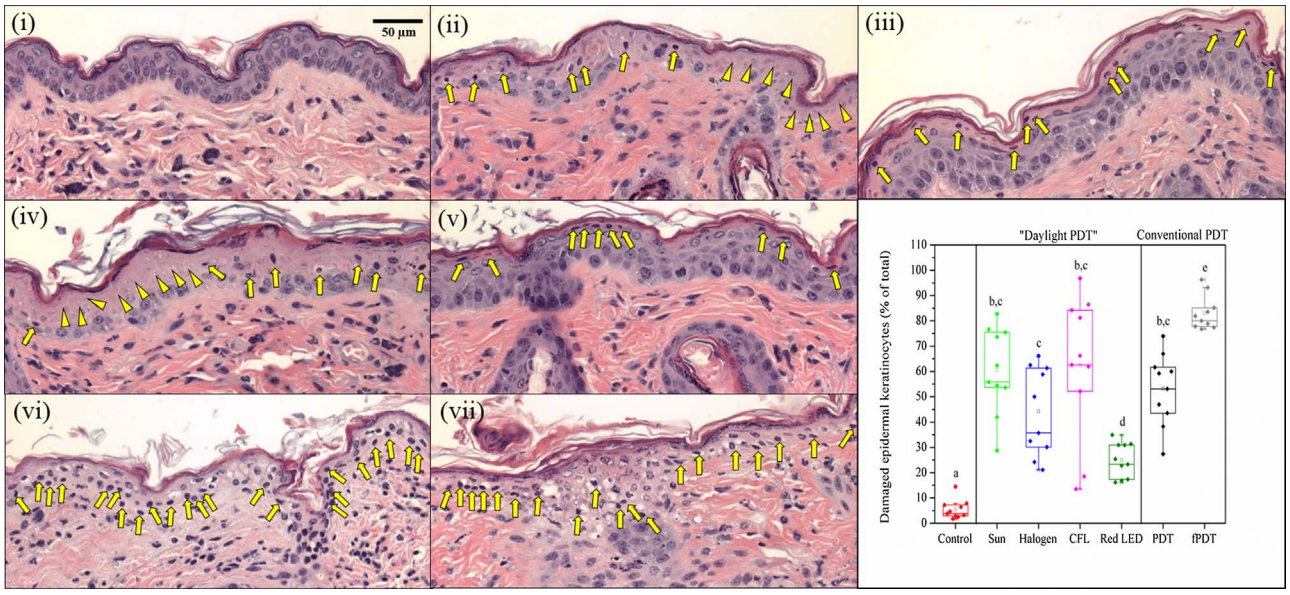

Fig. 4. Histological panel of PDT damage localized to the epidermis, representative of the group. Arrows indicate apoptotic cells; arrowheads indicate regions of necrosis. (i) Control; (ii) Natural sunlight; (iii) Halogen; (iv) CFL; (v) Red LED; (vi) PDT; (vii) fPDT. Scale bar (50 µm) is representative of all images. Different letters above the box plots mean significant differences.

conventional or fractionated light PDT. The treatment cohorts used different light sources (including natural sunlight, CFL, halogen, and red LED) with a low fluence rate delivery, matching the clinical paradigm used for 2.5 h, and the results were evaluated for PpIX fluorescence, cross-linking of STAT3, and damage to epidermal keratinocytes as a predictor of PDT response. These assays were tested in this mouse model, because each is a self-calibrated assessment of the skin which can be applied to clinical trials, as a surrogate endpoint of PDT efficacy. So, taken as a whole, this study tested several experimental lamps and assays which can be next implemented in clinical trials.

The first step of this work evaluated if PpIX could be photoactivated by low-irradiance light sources. Although the excitation spectra are different for each light source used in this study, the results presented in Fig. 2 show that PpIX was effectively activated in each treatment modality, regardless of the fluence rate. This observation is consistent with the work performed by Wiegell and co-workers [7], who showed that there was little accumulation of PpIX during “daylight PDT” in one of their early clinical trials. The low correlation between PpIX fluorescence and PpIX-weighted light dose observed in our study could perhaps be due exceeding the minimum light dose that is required to activate all the PpIX present in the skin.

Since the oxygen available in the tissue is an important key factor required to ensure PDT outcome, several studies have examined the influence of fluence light rate on the photochemical oxygen consumption during PDT. Generally, they have shown a decrease in oxygen depletion and better treatment responses when a low fluence rate was used [33,34]. Interestingly, in the current study no significant differences in relative oxygen saturation and relative blood volume fraction after PDT were observed for both “daylight PDT” and conventional PDT.

In terms of a more direct biological damage assay, inactivation of STAT3, a pro-cancer mediator, has been associated with PDT efficacy. Previous studies have shown that the formation of STAT3 cross-linking is dependent on the amount of photosensitizer, delivered light dose, and, light fluence rate [25,26,35]. In a study performed by Rohrbach et al. [35], low light fluence rate HPPH-PDT induced higher STAT3 cross-linking in a head and neck tumor model. The authors correlated their results with the vascular shutdown caused in the beginning of the treatment with high light fluence rate, limiting the oxygen available to promote the photoreaction [35,36].

A significant increase in STAT3 cross-linking was seen in the fractionated PDT group (Fig. 3). This result is consistent with previous findings due to the dark interval between fractions during which oxygen and PpIX can be replenished [21,37–39]. There were no significant differences observed between “daylight” PDT treatment groups and conventional, single scheme PDT, although all groups were significantly different from control. There was, however, a PpIX-weighted light-dose-dependent trend that can be seen in Fig. 5, suggesting that STAT3 cross-linking could be a sensitive predictor of PDT damage. Future studies are warranted to investigate the high variability we observed in our limited sample size.

In order to use a more conventional assay of PDT effect, pathology stained H & E images were used to quantify the keratinocyte damage, assayed 1 day after PDT, comparing the sun and lamp groups to “conventional” PDT. As shown in Fig. 4, the damage was similar for all groups, except for the red LED low fluence rate treatment, which could be explained by having received the lowest PpIX-weighted light dose (Table 1). Similar to the STAT3 assay, damage to keratinocyte had a strong linear correlation to the PpIX-weighted light dose, and should be

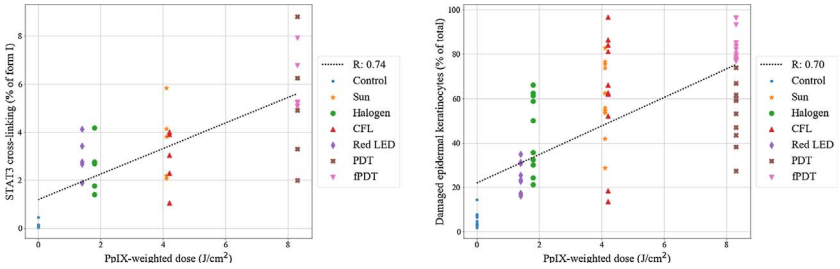

Fig. 5. Quantitative assessments of PDT-induced damage, as shown in Fig. 3 and 4, display strong linear correlation with each treatment modality’s respective PpIX-weighted light dose (Table 1).

investigated further in future studies as a reliable indicator of PDT outcome.

Taken together, the three bio assays described provide complementary information regarding PDT damage. One of the advantages of these in vivo biological assays is that comparisons can be made to an objective biological outcome, even when the light sources might not have the same effective light dose rate as weighted by the PpIX excitation spectrum. This is quite important given the complexity of adjusting light dose rates weighted through the action spectrum of PpIX, and how hard it is to accurately match these between different light sources and irradiation conditions. The keratinocyte assay proved the most practical for superficial, epidermal damage only. STAT3 cross-linking likely presented wider variability due to the nature of the biopsy containing the full thickness of the skin, and possibly due to the lower sample numbers taken. This assay and the logistics of it are labor intensive albeit directly sample the biological damage in the tissue. Finally, the PpIX fluorescence data can complement these biological assays through its two different excitation lasers — the blue laser has a shorter wavelength and provides more superficial information while the red excitation laser can penetrate deeper tissues.

5. Conclusions

In summary, since “daylight PDT” could be as effective as a single ALA-PDT illumination scheme, and there has been exponential growth in the number of studies using this, there is a need to objectively compare how well different irradiation schemes and light sources affect PDT outcome. Assessment of photophysical, biochemical, and biological damage can be achieved by the three biometric assays described – photoactivated bleaching of PpIX, epidermal keratinocyte damage, and cross-linking of STAT3 protein. Each of these assays gave similar results qualitatively that followed the trend: fPDT greater than all other treatment modalities, and low irradiance groups as effective as conventional (single illumination scheme) PDT. This study was somewhat limited by the variation in PpIX-weighted light doses, but was encouraged by the observation that CFL and sun in particular could still achieve similar effects to conventional PDT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pdpdt.2017.10.005.

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