5-ALA Photopreparation Using Pulsed NIR Enhances Skin Fluorescence via Temperature-Independent Cell Signaling Pathways

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ABSTRACT

The effect of near infrared light (940 nm) on the conversion of 5-aminolevulinic acid (5-ALA) to PpIX, a compound involved in photodynamic therapy (PDT), was examined. The back skin of three test subjects was irradiated with continuous wavelength and pulsed infrared light at 940 nm. These irradiations took place 50-53, 24-29, and 8-14 hours prior to the application of the 5-ALA. After a three-hour incubation period with 5-ALA, a FluoDerm™device was used to measure the fluorescence of the skin (emitting wavelength: 400-420 nm; measuring excitation wavelength: 610-720 nm), a direct indication of the activity of 5-ALA. 5-ALA must penetrate the skin and then be converted to PpIX before any fluorescence increase can be observed. Results: For two patients (one was disqualified), the continuous wavelength, 50 hour pre-irradiation condition, the FluoDerm readings showed a 19 to 23% increase in fluorescence ($p = 0.05$) compared to the no-irradiation, 5-ALA only control.

Keywords: Photopreparation, PDT, Photodynamic Therapy, photobiomodulation, PBM, 5-aminolevulinic acid, 5-ALA, photosensitizer, infrared, LLLT.

1. INTRODUCTION

An alternative approach in the treatment of skin lesions (Actinic Keratosis, Basal Cell Carcinoma, Acne, etc.) is photodynamic therapy (PDT). It uses light and aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) production to eradicate such lesions. PpIX formation is dependent on ALA percutaneous penetration and conversion. ALA-induced PpIX production in human skin is located in the epidermis but also in sebaceous glands and hair follicles [1]. Photoactivation within a specific range of wavelengths of this photosensitizer has been shown to lead to eradication of specific lesions. This approach, named photodynamic therapy (PDT), is based on photochemical reactions mediated through the interaction of photosensitizing agents, light, and oxygen, during which cytotoxic reactive oxygen species are formed causing damage (necrosis, apoptosis) to the target structures. Topical PDT has been found to be efficacious in the treatment of actinic keratosis, basal cell carcinoma, and acne vulgaris with various light sources, treatment regimens, and photosensitizers [2]. ALA–PDT efficacy is limited by the ability of ALA to penetrate the skin and induce PpIX accumulation. In an earlier study, we investigated the impact of pre-treating skin by increasing skin temperature using the radiant heat from near infrared light radiation to circumvent these limitations, enhancing clinical outcomes of patients with inflammatory type acne [3]. The study showed that, when skin was pre-heated with IR radiation, significant additional benefits over the nonheated side were observed for inflammatory and noninflammatory lesions, as well as in acne global severity scores 4 weeks post-treatment.

In the present study, we investigate the temperature-independent effects of infrared photopreparation in which photobiomodulation (PBM) may enhance PDT if applied a few hours/days prior to photoactivation with red light.

2. MATERIALS AND METHODS

2.1 Patient Selection

Three healthy patients (age 25, 49 and 56) with different skin types were recruited. The subjects were categorized on the Fitzpatrick Classification System. The first subject was phototype I and the two others were phototype III.

2.2 Study Procedure

Delineated treatment areas were drawn on each subject's back. These spaces had to be close enough together to enable simultaneous irradiation downstream in the experiment. Areas of each patient's back were then irradiated according to the predetermined photopreparation treatment parameter at the appropriate pre-5-ALA application and activation time (Figure 1). In this study, two light pulsing options were compared – pulsed versus continuous. Both options were set at 940 nm. The time set between the photopreparation treatment and the 5-ALA application was the other variable tested. Hence, for both pulsed and continuous modes, 50-53, 24-29, and 8-14 hour pre-treatments were performed on the appropriate back skin area. Table 2 shows the different areas drawn on each subject's back with the specific photopreparation option and pre-treatment time. Following the appropriately timed photopreparation treatment of the correct back skin area, 20% 5-aminolevulinic acid (Levulan Kerastick, Dusa, Wilmington, USA) was applied to the patient's back. After the topical application, an incubation time of 3 hours was set to optimize the transformation of the medication to protoporphyrin IX (PpIX). Finally, using the Fluoroderm device, the fluorescence of each subject's back areas was measured. The recorded data was statistically analyzed.

2.3 Materials

Besides the 20% 5-aminolevulinic acid (Levulan Kerastick, Dusa, Wilmington, USA) used as the photosensitizer and the FluoDerm (Dia-Medico, Denmark) to quantify the fluorescence of each patient's back, a prototype able to irradiate the back skin areas with pulsed (D50 pulsing condition) and continuous wavelength (CW) light at 940 nm was built. This machine was assembled at RoseLab Skin Optics Research Laboratory (Montreal, Canada). The D50-940 nm condition was made up of 940 nm light with a pulsing pattern consisting of a 50% duty cycle delivered in the repeating pattern (in µseconds where PD, PI, and PTI mean pulse duration, pulse interval, and pulse train interval, respectively) of: PD 500 on, PI 150 off, PD 500 on, PI 150 off, PD 500 on and PTI 1550 off (Figure 3). The irradiance delivered for all conditions (D50 and CW) was 75 mW/cm^2 and the fluence delivered for all conditions was 67.5 J/cm^2 (940 nm LED treatment duration D50 = 30 min and CW = 15 min).

2.4 Statistical analysis

The FluoDerm measured each tested condition. These measurements were then compared to the control (only 5-ALA without photopreparation). The means of these proportional changes were calculated. The final results were categorized by every photopreparation condition (50-53, 24-29, and 8-14 hours pre-treatment). A 95% confidence interval was calculated for each mean.

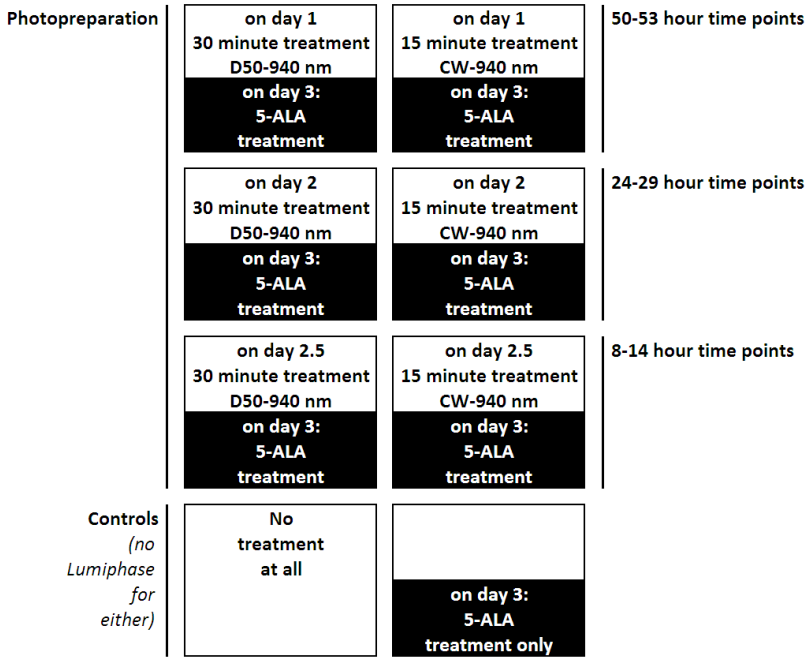

Figure 1: Representation of the delineated areas on a subject's back.

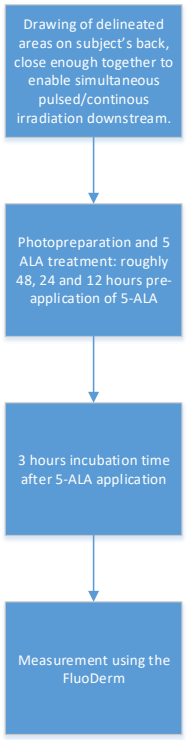

Figure 2. Study Protocol.

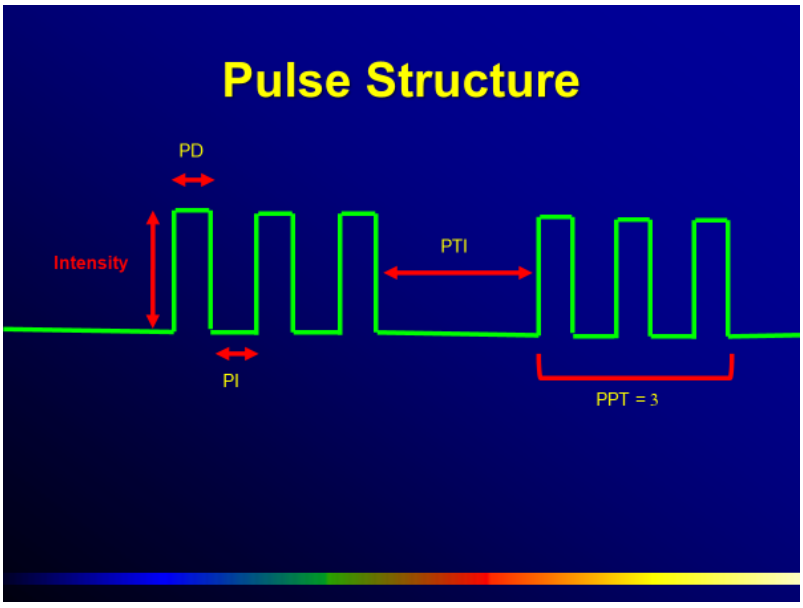

Figure 3. Sequential pulsing pattern definition: Pulse duration (PD) – LIGHT ON, Pulse interval (PI) – LIGHT OFF, Pulse per train (PPT) = # of pulses / train, Pulse train interval (PTI) – LIGHT OFF (longer dark zone/period).Table 2. Representation of the delineated areas on a subject's back.

3. RESULTS

The data is analyzed and presented as X-fold changes (or differences) as compared to the just 5-ALA control (Table 1). Taking any of the results as an example, the 8-14 hour time point using CW-940 showed a statistically significant 17% increase in fluorescence compared to the control. There is a large standard deviation of 0.30 and, as such, a large 95% confidence interval which translates into a range of X-fold changes from 0.75 to 1.58. So, clearly this result, although statistically significant, at p = 0.05, like all of the others, shows nothing of interest. It spans a range from a value showing less florescence to a value showing more florescence than the control. All of the results, except for the 50-53 hour time point using CW-940, are essentially the same in that they cover the no change (1.00) case. This exception showed a 19 to 23% increase in fluorescence (p = 0.05) compared to the no-irradiation, 5-ALA only control.

We started the study with 3 subjects and decided to discard a non-responder. Unexpectedly, he was not fluorescing at all. After investigation, we realized that the subject had significant alcohol intake the evening following the LED irradiation.

Table 1. Results.

			Subject "GC"		Subject "DB"		Means of X Fold Difference Values Across Biological Replicates	Standard Deviation	95% Confidence Interval	The 95% Confidence Interval Range	
			Fluoderm Reading	Proportional Change	Fluoderm Reading	Proportional Change					
Conditions	50-53 hour	D50-940	46.5	1.01	35.0	0.95	0.98	0.05	0.06	0.91	1.04
	time points	CW-940	55.0	1.20	45.0	1.22	1.21	0.01	0.02	1.19	1.23
	24-29 hour	D50-940	52.0	1.13	36.5	0.99	1.06	0.10	0.14	0.92	1.20
	time points	CW-940	47.0	1.02	48.0	1.30	1.16	0.19	0.27	0.89	1.43
	8-14 hour	D50-940	49.0	1.07	44.5	1.20	1.13	0.10	0.13	1.00	1.27
	time points	CW-940	44.0	0.96	51.0	1.38	1.17	0.30	0.41	0.75	1.58
	Control	Just 5-ALA	46.0		37.0						

4. DISCUSSION

It has been known for a long time that raising tissue temperature enhances the conversion of 5-ALA to PpIX during PDT. The application of an "outside-in" heat source like a bean bag or heating pad on the skin is enough to provide this effect, leading to better clinical results. What if a heat source would allow for superior, more uniform innovative “inside-out” pre-heating. Actually, NIR light can penetrate the deep dermis. It is absorbed by water which can raise the skin temperature up to 42ºC. This method called photopreparation, when NIR is applied 15 minutes prior to 5-ALA application, has been shown to increase the conversion of 5-ALA to PpIX, ultimately improving PDT anti-acne effects [3]. Based on this photopreparation thermal method, we decided to extend the concept to search for a non-thermal preconditioning mechanism of action. By extending the delay between NIR irradiation of skin and the application of 5-ALA to several hours/days, the temperature increase would have time to dissipate allowing for the measurement of non-thermal preconditioning effects like in photoprevention.

4.1 Photoprevention

The preventive effect of PBM (called photoprevention) with respect to UVB has been demonstrated *in vitro* and *in vivo* with red (660 nm) or near IR wavelengths to prevent sunburn (“light before the storm” effect) [4]. The proposed mechanism of action of this preconditioning method is related to p53 signaling pathway anti-apoptotic effects. It has been formerly reported that IR inhibited UVB-induced apoptosis *in vitro* by modulating the Bcl2/Bax balance, pointing to the role of p53 without heat shock protein (Hsp72-70) induction [5, 6]. Another study reported *in vivo/in vitro* results on pig skin and quantitative PCR (RT-PCR) data in which LEDs at 940 nm (10 mW/cm^2, 4 J/cm^2) 24h pre-UVB exposure abrogated the expected gene expression in primary human fibroblasts 24h post-UVB. Collagen type I and p53 were upregulated and MMP-1 downregulated as if it had not been exposed to UVB 24 h earlier [7]. By analogy with nature, the sun's most abundant IR wavelengths in the morning are likely preparing the skin for the detrimental UV rays at noon, at the zenith [8]. As a precursor to the day's coming UV insults to the skin, the ratio of UV to IR-A, as measured in the tropics, is lower in the morning and at the end of the day. Furthermore, post-inflammatory hyperpigmentation and scars can be prevented using specific parameters in the NIR spectrum. In this study, this preconditioning method was tested to enhance the non-thermal conversion of 5-ALA to PpIX. Several enzymes are involved in the production of

PpIX (figure 4). Presumably, one can precondition this pathway when exogenous 5-ALA is applied. It may involve NADPH or some enzymes like ferrochelatase. Anand et al. showed that you may enhance PDT treatment efficacy and tumor cell selectivity of δ-aminolevulinic acid (ALA)-based photodynamic therapy (PDT) via pretreatment of cells and tumors with methotrexate to enhance intracellular photosensitizer levels [9]. PpIX enhancement correlated with changes in protein expression of key porphyrin pathway enzymes, roughly a 4-fold increase in coproporphyrinogen oxidase and stable or slightly decreased expression of ferrochelatase.

Since a decrease in NADPH may increase PpIX level (and fluorescence), it is more than likely that PBM can modulate this pathway. A study by Ribeiro et al. showed that administration of red and infrared laser therapy (PBM) at different times positively modulates the activity of antioxidant enzymes (via the consumption of NADPH) and reduces stress markers during the muscle repair process [10]. Conversely, another team demonstrated that PBM regulated redox homeostasis as evidenced by enhanced NADPH levels and decreased NADP/NADPH ratio [11].

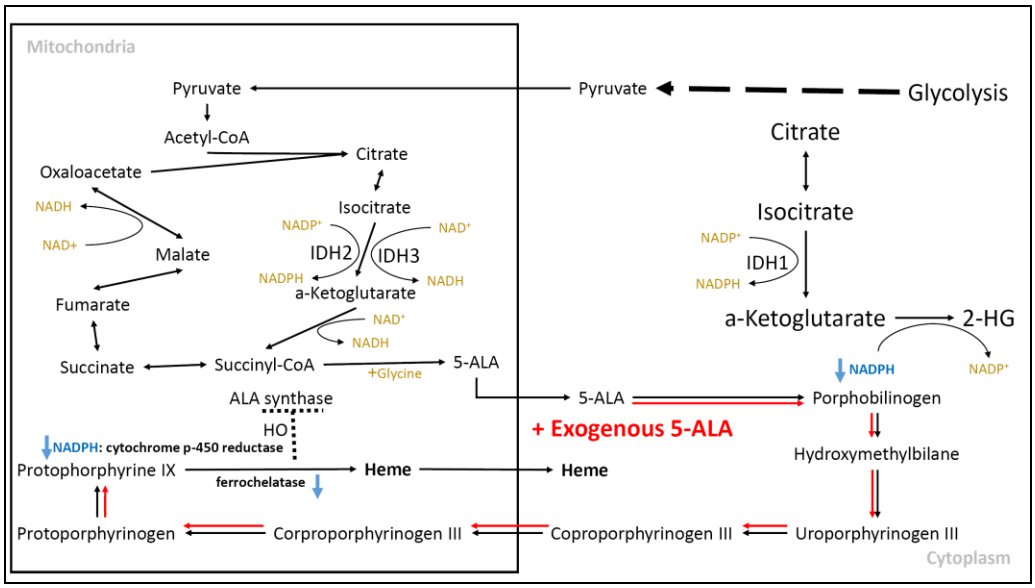

Figure 4. Porphyrin pathway enzymes. Exogenous 5-ALA route is presumably enhanced via NIR photobiomodulation of specific enzymes to ultimately increase PpIX.

Former PBM *in vitro* / *in vivo* studies show that it takes several hours (delay period) before you may get photobiomodulatory effects post irradiation using visible or NIR wavelengths. In fact, most PBM *in vitro* models' measurements (correlating with gene expression) are performed 24h post-irradiation. In the present study, the delay was extended to several days. Surprisingly, the 50-53 hour preconditioning delay demonstrated the best response (significant increase in fluorescence).

4.2 Pulsing

Past studies have shown that the pulsed light delivery mode may be superior to the continuous wave delivery mode, in particular with regard to healing and the production of collagen type I [12]. In addition, sequential light pulses, repeated sequences of short pulses followed by long intervals, appear to be superior to regular light pulses both *in vitro* [13] and clinically [14]. Work is currently underway to develop pulse codes tailored to each cutaneous presentation, e.g. inflammation, scarring, hyperpigmentation, prevention and preconditioning (like in this study), like a Morse Code to optimize PBM treatment. One condition involving the use of 940 nm with sequential pulsing at D50 (duty cycle 50%), 8-14 h prior to 5-ALA application showed a significant increase in fluorescence.

4.3 Alcohol intake

One of our volunteers did not show any increase in fluorescence following NIR preconditioning pre-PDT. We found out that he had ingested alcohol (9 beers) the evening following the NIR exposure. This may explain the lack of response to NIR preconditioning. Alcohol augments the inducibility of delta-aminolevulinic acid synthase. Alcohol has many biochemical and clinical effects on porphyrin and heme synthesis both in humans and laboratory animals. Ethanol suppresses the activity of porphobilinogen synthase (synonym: delta-aminolevulinic acid dehydratase), uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, and ferrochelatase, whereas it induces the first and rate-limiting enzyme in the pathway, delta-aminolevulinic acid synthase and also porphobilinogen deaminase [15].

The main weakness of this pilot study is the small number of patients (n=3).

5. CONCLUSION

Combination therapy using preconditioning exposure to non-thermal NIR photobiomodulation followed by ALA-PDT should be further investigated as a new combination modality to enhance efficacy and selectivity of PDT.

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