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Hemoporfin-mediated photodynamic therapy on normal vasculature: implications for phototherapy of port-wine stain birthmarks

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Hemoporfin-Mediated Photodynamic Therapy on Normal Vasculature: Implications for Phototherapy of Port-Wine Stain Birthmarks
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Dear Dr. Choi,

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at http://www.jctres.com/en/author-guidelines/ under "Manuscript preparation." Also, please use the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Aug 30, 2016.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a

menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

*******Reviewers' comments*******

Reviewer #1: The authors present a study on hemoporfin-mediated photodynamic therapy on normal vasculature in 23 Mice using a dorsal skinfold chamber model.
The experiments are original and the methods used to assess outcomes seem appropriate and were used previously in other studies.

I would like to ask the authors to clarify some issues.
* To the reader it might not be clear that 532 nm laser exposure without photosensitizer has not a therapeutic effect in this setting. The 532 nm laser is known to have therapeutic effects on its own when used with a pulse duration short enough to achieve photothermal interaction. In this study a very low power and long irradiation time is used, which should prevent any effect of the irradiation on its own. Authors should clarify this issue as in this study there are no experiments with 532 nm exposure alone.
* One disadvantage of using 532 nm light for excitation is the rather limited penetration depth at this wavelength. From histological studies in PDL treated port wine stains we know that the most resistant parts are the deeply located vessels and the small vessels. Are there absorption peaks at higher wavelength?
* P5 line6: Reference 1 is not from the original article. Moreover, the number of 95% of PWS located in the face is probably from an older publication with a high selection bias. In previous times, patients with PWS not located in the face were less likely to seek medical advice and treatment. In recent times this trend changes and we see much more PWS off the face. I would advice to look for a more recent publication.

Generally, the paper is well structured and well written and the discussion is appropriate in highlighting limitations and putting the results into a perspective.

Reviewer #2: The authors have performed important translational work on PDT in the context of PWS treatment, for which the standard therapy (PDL) is associated with suboptimal clinical results in a considerable fraction of patients. Accordingly, there is a clear medical need to optimize treatment for the patients with no alternative treatment options. The study attempts to meet that need through preclinical research using a standardized animal model and analytical techniques and, most importantly, clinically relevant endpoints.

The editorial board of JCTR is interested in publishing the study.

Before the work can be accepted for publication, we have the following suggestions to further improve the manuscript.

1. In the abstract, please specify that the dose dependence pertains to cumulative radiant exposure (and not e.g., photosensitizer dose).

2. Please provide a rationale for not including a 0 J/cm2 control in the study (at least, in Figure 3 there is no corresponding data point). In my own experience with the dorsal skin fold chamber in hamsters (>500 hamsters) I found that in some animals the placement of the windows can in itself cause hemostasis in the dorsal microcirculation. This would affect the entire data set. If you have any (historical) data sets that would support the absence of any perturbations in flow by the surgical procedure, please include this in the current data or refer to previous work. This will make your data stronger.

3. Please denote the protocol number assigned by the IRB to your animal study (section 2.1).

4. Please indicate the supplier of the Hemoporfin (section 2.2).

5. Details on animal anesthesia should be included. This is critical because some forms of anesthesia (e.g., ketamine/xylazine) induce respiratory acidosis, which will have an effect on systemic pO2 levels and therefore PDT efficacy.

6. The number of animals used per irradiation time should be explicitly mentioned (I presume N = 1 per cumulative radiant exposure).

7. From section 2.6, it is unclear how the binary analysis was performed exactly. Was a score of 1 (persistent vascular shutdown) assigned when all blood vessels in the optical window were hemostatic? How was the situation scored when half of the blood vessels were occluded by PDT?

8. In the discussion, the authors make a very important statement regarding the shutdown of small microvessels by PDT, which is clinically not achieved by PDL. In fact, the poor targeting of the smaller-diameter PWS vasculature has been a longstanding problem in therapeutic recalcitrance to PDL therapy. Accordingly, it would benefit the study if the authors could do a subanalysis on the extent of vascular shutdown as a function of vascular diameter. The brightfield images in Figure 1 and 2 clearly show that the vessels in a single field of view comprise different diameters, whereas the hemodynamic affliction differs between vessels in a single animal subjected to PDT. So, the existing data set could be re-analyzed to empirically yield credence to the claim that smaller vessels are treated equally well as larger vessels, which could then be present as an important clinical benefit of PDT over PDL treatment.

9. In the Discussion, the authors state that the cumulative radiant exposure threshold to achieve vascular occlusion by PDT was ~4-fold lower for NPe6 compared to Hemoporfin. This statement is only valid if the administered concentration was comparable, so please elaborate on the dosages in the discussion.

10. Our group has shown that laser-induced changes in hemodynamics occur directly after laser irradiation and last for up to an hour after lasing [Opt Express. 2005 Feb 7;13(3):702-15; Optics Express 2007:15:8493-8506; J Dermatol Sci. 2011 Sep;63(3):139-47]. These changes entail both the photothermal response and the hemodynamic response. The vascular occlusion in PDT is caused by hyperoxidative damage to endothelium and consequent thrombosis, which very are essentially comparable to the hemodynamic response that ensues laser irradiation (selective photothermolysis). What I don't understand is that the vascular shutdown in some cases takes days to complete (e.g., Figure 1B and 2C). Can the authors provide an explanation about the mechanism of vascular shutdown in their model?

Authors' rebuttal:

We greatly appreciate the reviewers' time to read and comment on the submitted manuscript. Please find our responses below to each of the comments.

Reviewers' comments:

Reviewer #1:

* To the reader it might not be clear that 532 nm laser exposure without photosensitizer has not a therapeutic effect in this setting. The 532 nm laser is known to have therapeutic effects on its own when used with a pulse duration short enough to achieve photothermal interaction. In this study a very low power and long irradiation time is used, which should prevent any effect of the irradiation on its own. Authors should clarify this issue as in this study there are no experiments with 532 nm exposure alone.

The text has been modified and now reads:

2.3 Laser irradiation

For Hemoporfin-mediated PDT, we utilized a diode-pumped solid-state laser (532nm, Dragon Laser, Jilin, China) at an irradiance of 100mW/cm^2. We varied the irradiation time (0 to 5500s) to achieve radiant exposures ranging between 0 and 550J/cm^2. A low irradiance was used to mitigate potential photothermal effects of the 532nm excitation alone on the microvasculature.

* One disadvantage of using 532 nm light for excitation is the rather limited penetration depth at this wavelength. From histological studies in PDL treated port wine stains we know that the most resistant parts are the deeply located vessels and the small vessels. Are there absorption peaks at higher wavelength?

There are other absorption peaks at higher wavelengths, however these peaks are much smaller in magnitude. From this study, published by Lei et al, Hemoporfin has absorption peaks at 570 nm and 620 nm, but the absorption is 20-50% less compared to that at 532 nm.

(http://www.ncbi.nlm.nih.gov/pubmed/22959803)

We selected 532nm primarily because it is currently used clinically with Hemoporfin (reference 16).

* P5 line6: Reference 1 is not from the original article. Moreover, the number of 95% of PWS located in the face is probably from an older publication with a high selection bias. In previous times, patients with PWS not located in the face were less likely to seek medical advice and treatment. In recent times this trend changes and we see much more PWS off the face. I would advice to look for a more recent publication.

Due to the comment about selection bias, we have decided not to report on a specific number for incidence rate on the face; instead, we have adjusted the text and citation to read:

1. Introduction

Port wine stain (PWS) birthmarks are congenital vascular malformations that are typically found on the face and neck regions [1]. Current treatment protocols in the United States involve the use of a pulsed dye laser (PDL) combined with cryogenic cooling of the skin [2]. Yellow light, in the 585–595nm wavelength range, is strongly absorbed by intravascular hemoglobin and can photocoagulate the targeted vasculature. Unfortunately, light in this spectral range is also strongly absorbed by epidermal melanin. This competitive absorption limits the light available for absorption by the targeted vasculature, and hence limits the efficacy of PDL treatment. To this end, a need exists to evaluate alternate approaches to treat PWS vasculature in a safe and effective manner.

1. Jacobs, A.H. and R.G. Walton, *The incidence of birthmarks in the neonate.* Pediatrics, 1976. **58**(2): p. 218-222.

Generally, the paper is well structured and well written and the discussion is appropriate in highlighting limitations and putting the results into a perspective.

Reviewer #2:

1. In the abstract, please specify that the dose dependence pertains to cumulative radiant exposure (and not e.g., photosensitizer dose).

The text has been modified and now reads:

Results. We observed four general hemodynamic responses to PDT: (1) At low radiant exposures, we did not observe any persistent vascular shutdown; (2) at intermediate radiant

exposures, we observed delayed vascular shutdown effect with significant change to the vascular structure; (3) at intermediate radiant exposures, we observed an acute vascular shutdown effect with gradual restoration of blood flow and no significant changes to the vascular structure; and (4) at high radiant exposures, we observed acute vascular shutdown that persisted during the entire 7-day monitoring period, with no change in vascular structure. With light dose–response analysis, we estimated a characteristic radiant exposure of 359 J/cm^2 that was required to achieve persistent vascular shutdown observed on Day 7 after PDT.

2. Please provide a rationale for not including a 0 J/cm2 control in the study (at least, in Figure 3 there is no corresponding data point). In my own experience with the dorsal skin fold chamber in hamsters (>500 hamsters) I found that in some animals the placement of the windows can in itself cause hemostasis in the dorsal microcirculation. This would affect the entire data set. If you have any (historical) data sets that would support the absence of any perturbations in flow by the surgical procedure, please include this in the current data or refer to previous work. This will make your data stronger.

We routinely perform control 7 day experiments without any phototherapy. The injection of the photosensitizer without 532 nm irradiation does not induce persistent vascular shutdown. We have now included a data point of "0" with 0 J/cm^2, which did not affect the characteristic radiant exposure value RE$_{50}$/7. We updated the number of experiments to 24 in several locations within the manuscript to account for this added data point.

Furthermore, the consistent absence of persistent vascular shutdown at lower radiant exposures suggests that hemostasis induced by the titanium chambers themselves, did not play a confounding role in our analysis.

3. Please denote the protocol number assigned by the IRB to your animal study (section 2.1).

The text has been modified and now reads:

2.1 Dorsal window chamber model

Similar to previous work [8, 11, 13], we utilized the mouse dorsal window chamber model installed on adult C3H mice (25-30 g, n = 24). The Institutional Animal Care and Use Committee at University of California, Irvine approved the *in-vivo* experiments (IACUC protocol number 2002-2339).

4. Please indicate the supplier of the Hemoporfin (section 2.2).

The text has been modified and now reads:

Hemoporfin (Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Company, Shanghai, China) was reconstituted with saline into a stock solution of 25 mg/mL. The drug dose (1mg/kg) was selected based on clinical treatment parameters used in previous publications [4 ,5, 6].

5. Details on animal anesthesia should be included. This is critical because some forms of anesthesia (e.g., ketamine/xylazine) induce respiratory acidosis, which will have an effect on systemic pO2 levels and therefore PDT efficacy.

The text now reads:

2.4 Experimental design

We anesthetized and positioned the animal in a custom window chamber holder placed on top of a heating pad, identical to previously reported studies (8, 11, 13). A mixture of isoflurane and oxygen was used as anesthesia. We administered Hemoporfin (2mg/kg) via retro-orbital injection and initiated laser irradiation of the epidermal side of the window chamber immediately after injection. Irradiation times were randomized to minimize systematic bias. In total, we performed 24 experiments, with n = 1 per cumulative radiant exposure.

6. The number of animals used per irradiation time should be explicitly mentioned (I presume N = 1 per cumulative radiant exposure).

This has been modified and now reads.

2.4 Experimental design

We anesthetized and positioned the animal in a custom window chamber holder placed on top of a heating pad, identical to previously reported studies (8, 11, 13). We administered Hemoporfin (2mg/kg) via retro-orbital injection and initiated laser irradiation of the epidermal side of the window chamber immediately after injection. Irradiation times were randomized to minimize systematic bias. In total, we performed 24 experiments, with one experiment per cumulative radiant exposure.

7. From section 2.6, it is unclear how the binary analysis was performed exactly. Was a score of 1 (persistent vascular shutdown) assigned when all blood vessels in the optical window were

hemostatic? How was the situation scored when half of the blood vessels were occluded by PDT?

For a score of 1, all observed blood vessels within the window chamber were evaluated as hemostatic. If only half of the blood vessels were occluded, the score was evaluated as 0. We have modified the text and it now reads:

2.6 Dose response analysis

Similar to previous publications [8, 11], we used the approach of dose-response analysis with a longitudinal study design. Raw speckle images were collected at the following time points: pre-PDT; immediately post-PDT; and at Days 1, 2, 3, and 7 after PDT. All authors independently reviewed the SFI images collected on Day 7 of each experiment and graded each image on a binary scale: a "0" (no persistent or incomplete vascular shutdown on Day 7) or "1" (persistent vascular shutdown on Day 7) was assigned to each Day 7 image. We used a commercial software package (Prism version 5.0d, GraphPad Software, San Diego, CA) to apply a sigmoidal fit to the data and estimate a characteristic radiant exposure ($RE_{50}/7$) at which persistent vascular shutdown was observed on Day 7 after PDT.

8. In the discussion, the authors make a very important statement regarding the shutdown of small microvessels by PDT, which is clinically not achieved by PDL. In fact, the poor targeting of the smaller-diameter PWS vasculature has been a longstanding problem in therapeutic recalcitrance to PDL therapy. Accordingly, it would benefit the study if the authors could do a subanalysis on the extent of vascular shutdown as a function of vascular diameter. The brightfield images in Figure 1 and 2 clearly show that the vessels in a single field of view comprise different diameters, whereas the hemodynamic affliction differs between vessels in a single animal subjected to PDT. So, the existing data set could be re-analyzed to empirically yield credence to the claim that smaller vessels are treated equally well as larger vessels, which could then be present as an important clinical benefit of PDT over PDL treatment.

This is an excellent point. Within the field of view that we observe, we are less sensitive to capillary blood flow. With the data collected in this study, we are hesistant to make any substantive claims with regards to small microvessels as our imaging field of view and exposure time were selected to focus on the larger microvessels (arterioles and venules). We now have adapted our microscope to laser speckle imaging, which will allow us to look at small microvessels. This is a topic of future work.

9. In the Discussion, the authors state that the cumulative radiant exposure threshold to achieve vascular occlusion by PDT was ~4-fold lower for NPe6 compared to Hemoporfin. This statement is only valid if the administered concentration was comparable, so please elaborate on the

dosages in the discussion.

The same drug concentrations and radiant exposures were used, compared to our previously reported data on NPe6. We have adjusted the text and now reads:

4. Discussion

The experimental data collectively suggest that Hemoporfin-mediated PDT can achieve persistent vascular shutdown at a characteristic light dose of 359 J/cm^2, using methodology similar to previous published clinical studies [5, 15, 16]. At high radiant exposures (>360 J/cm^2), Hemoporfin-mediated PDT consistently induced persistent vascular shutdown. In our previous studies [8, 11] involving NPe6-mediated PDT, we identified a characteristic radiant exposure of 85J/cm^2 to achieve persistent vascular shutdown, which was equivalent to an irradiation time of 10min, at the same 1mg/kg drug dosage per mouse. In comparison, the characteristic radiant exposure of 359J/cm^2 associated with Hemoporfin-mediated PDT is equivalent to a treatment time of 60min, which may be too long for patients to tolerate in the clinic.

10. Our group has shown that laser-induced changes in hemodynamics occur directly after laser irradiation and last for up to an hour after lasing [Opt Express. 2005 Feb 7;13(3):702-15; Optics Express 2007:15:8493-8506; J Dermatol Sci. 2011 Sep;63(3):139-47]. These changes entail both the photothermal response and the hemodynamic response. The vascular occlusion in PDT is caused by hyperoxidative damage to endothelium and consequent thrombosis, which very are essentially comparable to the hemodynamic response that ensues laser irradiation (selective photothermolysis). What I don't understand is that the vascular shutdown in some cases takes days to complete (e.g., Figure 1B and 2C). Can the authors provide an explanation about the mechanism of vascular shutdown in their model?

Our current hypothesis is some photodamage induces apoptosis in the endothelial cells, which then leads to delayed cellular death, and potentially delayed hemodynamics. We have also observed this effect with other photosensiziters (BPD and NPe6). Similar to the response above, we believe that microscopic evalution, combined with histological analysis, will allow us to more precisely identify the mechanism for delayed vascular shutdown.

2nd editorial decision:

Date: 3-Sep-2016

Ref.: Ms. No. JCTRes-D-16-00021R1
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Phototherapy of Port-Wine Stain Birthmarks
Journal of Clinical and Translational Research

Dear Dr. Choi,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research