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# Histologic Changes Associated with Talaporfin Sodium-Mediated Photodynamic Therapy in Rat Skin

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## Abstract

**Background and Objective—**Alternative treatments are needed to achieve consistent and more complete port wine stain (PWS) removal, especially in darker skin types; photodynamic therapy (PDT) is a promising alternative treatment. To this end, we previously reported on Talaporfin Sodium (TS)-mediated PDT. It is essential to understand treatment tissue effects to design a protocol that will achieve selective vascular injury without ulceration and scarring. The objective of this work is to assess skin changes associated with TS-mediated PDT with clinically relevant treatment parameters.

**Study Design/Materials and Methods—**We performed TS (0.75 mg/kg)-mediated PDT (664 nm) on Sprague-Dawley rats. Radiant exposures were varied between 15–100 J/cm$^2$. We took skin biopsies from subjects at nine hours following PDT. We assessed the degree and depth of vascular and surrounding tissue injury using histology and immunohistochemical staining.

**Results—**TS-mediated PDT at 0.75 mg/kg combined with 15 and 25 J/cm$^2$ light doses resulted in vascular injury with minimal epidermal damage. At light dose of 50 J/cm$^2$, epidermal damage was noted with vascular injury. At light doses >50 J/cm$^2$, both vascular and surrounding tissue injury were observed in the forms of vasculitis, extravasated red blood cells and coagulative necrosis. Extensive coagulative necrosis involving deeper adnexal structures was observed for 75 and 100 J/cm$^2$ light doses. Observed depth of injury increased with increasing radiant exposure, although this relationship was not linear.

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**Conclusion—**TS-mediated PDT can cause selective vascular injury; however, at higher light doses, significant extra-vascular injury was observed. This information can be used to contribute to design of safe protocols to be used for treatment of cutaneous vascular lesions.

### Keywords

coagulative necrosis; depth of photo-injury; NPe6; photochemical; port-wine stains

## INTRODUCTION

In the United States, port wine stain birthmarks (PWS) typically are treated with high intensity pulsed light[1–6]. The pulsed dye laser (PDL, λ = 585–595 nm) combined with epidermal cooling of the skin is the most commonly used light source. Intravascular oxy- and deoxy-hemoglobin are selectively targeted by the PDL. The light energy is converted to heat, which can cause localized vascular damage. Activation of the clotting cascade, and subsequent formation of thrombi, can lead to a considerable reduction or complete shutdown of blood flow[7].

While PDL can lighten PWS, many laser treatment sessions are generally required (8 or more)[4,8–10]. Additionally, treatment of patients with darker skin is more difficult due to higher epidermal melanin content that competitively absorbs PDL light[11]. In these patients, the maximum safe radiant exposure is lower due to the risk of damage to the epidermis, which limits treatment efficacy.

A clinical need exists to develop alternate or adjuvant treatment protocols. Photodynamic therapy (PDT) is a promising treatment option for PWS that involves the collective use of a photosensitizing agent and light source to induce the formation of cytotoxic singlet oxygen[12–14]. PDT potentially enables photocoagulation of vessels of all sizes and at greater depths than PDL therapy, with considerably reduced risk of epidermal necrosis. This approach has been used most commonly in China with Photocarcinorin[15] or Hemoporfin[16] as photosensitizers. However, the photosensitive period is at least two weeks and ulceration and scarring are significant risks.

We have endeavored to design alternative PDT protocols with a shorter photosensitive period and fewer side effects. Talaporfin Sodium (TS) in combination with 664 nm light is a promising option. We previously characterized blood flow changes associated with TS-mediated PDT on C3H mice[17] over a period of seven days. At radiant exposures greater than 85 J/cm$^2$, we observed complete shutdown of blood flow, whereas with lower radiant exposures, blood flow either persisted or was temporarily shutdown before returning.

The type, extent and depth of tissue injury associated with TS-mediated PDT has not previously been evaluated. For TS-mediated PDT to be a clinically-viable therapeutic option, the injury should be limited to PWS vasculature that is located primarily over a depth of 1–2 mm from the skin surface.

We hypothesized that TS-mediated PDT can cause selective vascular damage and the depth of this damage will be dependent on light dose. To test this hypothesis, we administered

0.75mg/kg of TS to Sprague Dawley rats, and varied the light dose (15–100 J/cm$^2$) and assessed the degree and depth of vascular and surrounding tissue injury using Hematoxylin and Eosin staining and immunohistochemistry staining. In contrast with the higher drug dosage used in previous studies[17,18] with a different preclinical animal model, we used 0.75mg/kg TS to match the dose currently under study in a clinical trial.

## MATERIALS AND METHODS

### Sprague Dawley rat model

We performed experiments on adult male Sprague Dawley rats (200–250g, n=10). We report on data collected from eight subjects, as two died before completion of the experiments. All experiments were approved by the Institutional Animal Care and Use Committee at University of California, Irvine.

### Light source

We used a commercially available diode laser (664 nm, Biolitec AG) as the excitation source. A 1mm diameter fiber (P1000-2-UV-VIS, Ocean Optics) delivered light from the laser to a beam expander (BE10M-B, Thorlabs), which produced a uniform 50mm diameter collimated beam. A handheld power meter (Coherent PM3 device head, Molectron 3Sigma power meter) was used prior to irradiation to measure the optical power, and the power was adjusted to achieve an irradiance of 100mW/cm$^2$.

### Photosensitizer

We combined reconstituted TS (Light Sciences Oncology; Bellevue, Washington) with sterile saline to generate a stock solution of 25mg/mL. Prior to injection, the stock solution was further diluted into 1mL of sterile saline to achieve an administered dosage of 0.75mg/kg.

### Fiber-based fluorescence probe

To ensure that TS was successfully administered into the vasculature, we monitored real-time fluorescence emission of the circulating TS solution. A laser diode (660 nm, 50 mW, Roithner, ADL-66505TL) was coupled to the center fiber of a seven-fiber fluorescence/reflection probe (Ocean Optics, R400-7-UV/VIS). The surrounding six fibers were used for collection of the emitted fluorescence. Fluorescence emission was filtered using a razor-edge filter (664 nm, Semrock, LP02-664RU-18-D) placed in an inline fiber optic filter holder (World Precision Instruments, 56200). Filtered emission was coupled through a fiber (Ocean Optics, Dunedin, FL) directly into a spectrometer (Ocean Optics, USB 2000) and analyzed using OceanView software (Ocean Optics, v1.4.0). The spectrometer exposure time was 40 ms and data collection rate 0.5 Hz. Fluorescence data were analyzed by integrating the emission from 650–850 nm.

### PDT protocol and Microscopy

We used 5% isoflurane to induce anesthesia. The animal then was placed onto a heating pad for body temperature regulation and maintained under anesthesia with 3% isoflurane

(balance $O_2$). We shaved and applied a depilatory cream (Nair, Church & Dwight, Marietta, GA) to the dorsum of each animal and identified three regions of interest. We created a customized opaque mask containing three 10mm diameter circles, with each circle spaced 5mm apart in a triangular fashion (Figure 1). We used this mask to restrict PDT only to the regions of interest. We injected a 0.75 mg/kg TS solution via tail-vein injection into each subject. We placed the fluorescence probe onto the subject approximately 50mm away from the treatment site. Once the intravascular presence of the drug was confirmed by TS fluorescence emission, PDT irradiation was initiated within 30 seconds using the 664nm laser. For all experiments, we used an irradiance of 100mW/cm$^2$ and varied the irradiation time to achieve specific values of radiant exposure, 15, 25, 50, 75 and 100J/cm$^2$. Three treatment spots with varying radiant exposures per subject were achieved by blocking specific regions of interest at predetermined time points during laser irradiation. Histological observations were made from images collected from biopsy with a microscope (Zeiss Axioplan 2, Zeiss Plan-Neofluar objectives 20×/0.5 and 10×/0.3).

## Histology

We euthanized subject at a time point of nine hours after PDT. After euthanization, we collected 12mm diameter skin biopsies at each of the three treatment sites. A fourth site at which no light treatment occurred was biopsied to serve as a negative control. Biopsies included a border of normal, untreated skin surrounding the treatment region. Tissues were placed in 5% formalin and processed for histological analysis. Prior to embedding, the tissues were sliced through the middle of the sample and then embedded into paraffin wax. After embedding, three 10μm thick tissue sections were removed from the tissue block and stained with hematoxylin and eosin (H&E). Two board-certified dermatopathologists (JY, SD) reviewed the H&E stained slides of TS-mediated PDT treatment spots from each animal and depth of necrosis quantified.

For the 9-hour time point on day 0, immunohistochemistry stains for Caspase 3 and CD31 were applied to enable study of apoptosis and to identify vasculature, respectively. Caspase 3 is a known mediator of apoptosis that is expected to result from cytotoxic injury associated with reactive oxygen species generated during PDT[19,20]. To quantify a positive Caspase 3 stain, the dermatopathologists rated each slide using a integer scale of 0–3 to describe the color and quality of each stain: Grade 0: no staining, Grade 1: faint staining comparable to background non-specific staining of surrounding tissue; Grade 2: red, moderately intense staining; and Grade 3: bright red intense staining. To be considered a positive stain, the Capsase 3 antibody was associated with a strong diffuse-to-punctate cytoplasmic staining of Grade 2 or 3. Non-specific, negative staining was noted with weak Grade 1 staining intensity or non-cellular staining pattern such as staining of lumen of the vessels or stromal staining at the edge of the tissue sample. A minimum of five depth measurements were measured from each biopsy at each radiant exposure

# RESULTS

## Normal Control

The epidermis, dermis, and subcutaneous layers in the normal control had structural features characteristic of normal tissue (Figure 2). The epidermis had a 4–6 cell thick layer of stratified squamous epithelium. The dermis was comprised of thin wispy collagen at the superficial layer and uniformly distributed adnexal structures (i.e. hair follicles, adnexal glandular structures), surrounded by medium sized collagen consistent with normal dermis. In the deep dermis, a thin layer of stratum liposum, composed of aggregate of uniform adipocytes, was evident. The stratum fibrosum consisted of thicker caliber vessels embedded in a loose stroma composed of wispy collagen fibers. Non-specific Caspase 3 expression was noted throughout the upper dermis (Figure 3).

## 15 J/cm$^2$

At 15 J/cm$^2$ radiant exposure, the tissue exhibited minimal and limited structural changes from the normal control. Subcorneal micro-pustules, mild dermal edema, and focal epidermal erosion with related epidermal keratinocytic apoptosis were noted in all samples. The average depth of tissue necrosis was noted to be 0.12 mm from the top of the epidermis. At 9 hours, Caspase 3 expression was not observed.

## 25 J/cm$^2$

At 25 J/cm$^2$ radiant exposure, the epidermis showed minor erosion, which was more pronounced and widespread than at 15 J/cm$^2$ (Figure 2). Blood vessels in the dermis were ectatic and showed enlargement and swelling of endothelial cells. The average depth of tissue necrosis was 0.56 mm from the top of the epidermis. At 9 hours, Caspase 3 expression revealed specific staining of superficial vessels in the upper dermis (Figure 3). Non-specific staining was observed in vessels above the adipocytes (Figure 3).

## 50 J/cm$^2$

At 50 J/cm$^2$, ulceration of the epidermis into the mid dermis was observed (Figure 2). Ischemic changes of the entire follicular structure was noted. Adjacent blood vessels showed signs of structural distress and some intravascular thrombi were noted. Early vasculitis was noted with neutrophils in the wall of the small caliber vessels with focal fibrin deposition; however, minimal extravasated blood cells were observed, indicating that the blood vessels were mostly intact. There was a mild chronic inflammatory response and some neovascularization in the stratum fibrosum. The average depth of tissue necrosis was 1.64 mm. At 9 hours, Caspase 3 expression revealed specific staining of vessels in the stratum fibrosum. Non-specific staining was again observed in vessels above the adipocytes.

## 75 J/cm$^2$

At 75 J/cm$^2$, the epidermis was completely eroded and the dermis was further damaged (Figure 2D). The ischemic change involved the entire dermis through the level of the skeletal muscle. Dermal blood vessels were severely damaged. Pronounced vasculitis was noted with extravasated red blood cells. Significant neovascularization and a brisk fibroblastic

proliferation were noted, representative of a mild and early wound healing response. The average depth of tissue necrosis was 2.04 mm. At 9 hours, Caspase 3 expression was observed in vessels at the superficial dermis and near the stratum fibrosum, in addition to a higher density of Caspase 3 expression overall (Figure 3).

### 100 J/cm$^2$

At 100 J/cm$^2$, the epidermis was completely eroded and dermal necrosis extended into the skeletal muscles. In the dermis, the blood vessels were completely obliterated and extravasated red blood cells were pronounced. Prominent neovascularization and a significant fibroblast population were observed as well, indicating a brisk wound healing process. The average depth of tissue necrosis was 2.22 mm. At 9 hours, Caspase 3 expression was observed at the highest overall levels in vessels throughout the dermis.

## DISCUSSION

A clinical need exists to develop alternate protocols to treat PWS birthmarks. In our previous work (5, 6), we used the dorsal window chamber model to determine that TS-mediated PDT can induce a range of vascular-specific injury severity, including persistent vascular shutdown. With these promising preclinical findings related to treatment efficacy, we report here on safety considerations, and specifically the type, extent, and depth of tissue injury resulting from TS-mediated PDT.

At 15 J/cm$^2$, we observed minimal tissue damage to the epidermis but no significant vascular injury and no injury to the surrounding dermis. At 25 J/cm$^2$, we observed minimal tissue damage to the epidermis, while vascular injury was noted in the Caspase 3 stain in the upper portion of the dermis. No injury was observed in the surrounding dermis. There was a pronounced increase in tissue damage severity and depth of injury starting at 50 J/cm$^2$, which was further observed with increasing light dosage. Dermal fibrosis and granulation tissue were noted at the level of the stratum carnosus for all doses 50 J/cm$^2$ and above.

At the dose of 50 J/cm$^2$, epidermal erosion was observed followed by vascular damage in the form of endothelial swelling. Mild fibroblastic proliferation was observed, indicating dermal wound response. At 75 J/cm$^2$, epidermal erosion was pronounced over the treatment site. Vascular damage was noted in the form of endothelial swelling. There was proliferation of fibroblasts to the injury site. At 9 hours, Caspase 3 staining revealed selective deep vascular damage (deep dermis above adipocyte layer), with an increase in Caspase 3 staining of the stromal fibroblasts as a result of increased damage to the surrounding tissue. At 100 J/cm$^2$, the epidermis was completed eroded, followed by deep vascular damage through the stratum fibrosum. Significant surrounding tissue damage was noted in the form of ischemic necrosis.

At higher doses (75 and 100 J/cm$^2$) there was pronounced granulation tissue with prominent neovascularization highlighted by small caliber, single layer vessels with luminal red blood cells. Vascular destruction and necrosis and collagen degeneration also occurred. Neovascularization, the process by which new vessels are formed, was prominent in the dermis-stratum carnosus interface. At 75 J/cm$^2$, damage was observed at the mid stratum

carnosus level. At 100 J/cm$^2$, damage occurred from the epidermis through the stratum fibrosum. A high level of fibroblast proliferation was noted compared to the normal sample. Neovascularization was also prominent in the stratum fibrosum.

Collectively, our results indicate that the severity and depth of injury depends on light dose (Figure 4). This is generally thought to be the case, but has not been frequently demonstrated in the dermatologic literature with systemically administered photosensitizers and has not previously been demonstrated with TS for a cutaneous application. There was a direct relationship between the dose and the amount and grade of epidermal injury, vascular damage and extra-vascular dermal changes. While the average depth of injury was not linearly dependent on the light dosage, we observed a clear relationship between increasing depth and increasing radiant exposure. At radiant exposures of 75J/cm$^2$, we observed ischemic necrosis at depths greater than 2mm, which increases tremendously the risk of post-treatment scar formation.

These data, combined with the efficacy data from our previous work (5, 6), suggest that the TS-mediated PDT alone may not be viable for treatment of PWS birthmarks. PDT with hemoporphin derivatives is currently in use for treatment of PWS birthmarks in China[12,21]. While good results can be demonstrated, scarring can occur. We chose to evaluate TS mediated PDT rather than Hemoporfin mediated PDT because in a previously published report we demonstrated that that persistent vascular shutdown is possible with the latter phototherapy, but at a characteristic radiant exposure that was more than four times greater compared to the radiant exposure needed with TS mediated PDT. Combination protocols, such as PDT combined with PDL irradiation, may be a more suitable treatment approach, as they are expected to allow for use of relatively low PDT light doses and hence increase the margin for safety. Alternatively, for cutaneous PDT protocols with systemic photosensitizers, especially when the vasculature is targeted and there is risk of deep necrosis and scarring, safety measures should be developed. One example would be a method for non-invasive monitoring of tissue effects during the PDT procedure with determination of an objective endpoint that avoids injury to non-targeted tissue.

We note that the rat skin model differs from both regular human skin and skin with a PWS. The main differences include differences in keratinization, thickness, and the number and density of blood vessels found in the superficial dermis. Despite these differences, the rat model enables initial characterization of the effects of TS-mediated PDT at clinically relevant doses and a model with which we can perform comparative studies using different PDT and PDL treatment approaches.

In conclusion, we have identified a correlation between the administered radiant exposure and the depth and extent of tissue injury during TS-mediated PDT. Future studies are expected to identify possible mechanisms via use of additional immunohistochemistry stains, and optimization of the safety and efficacy of PDT and PDL treatment protocols.

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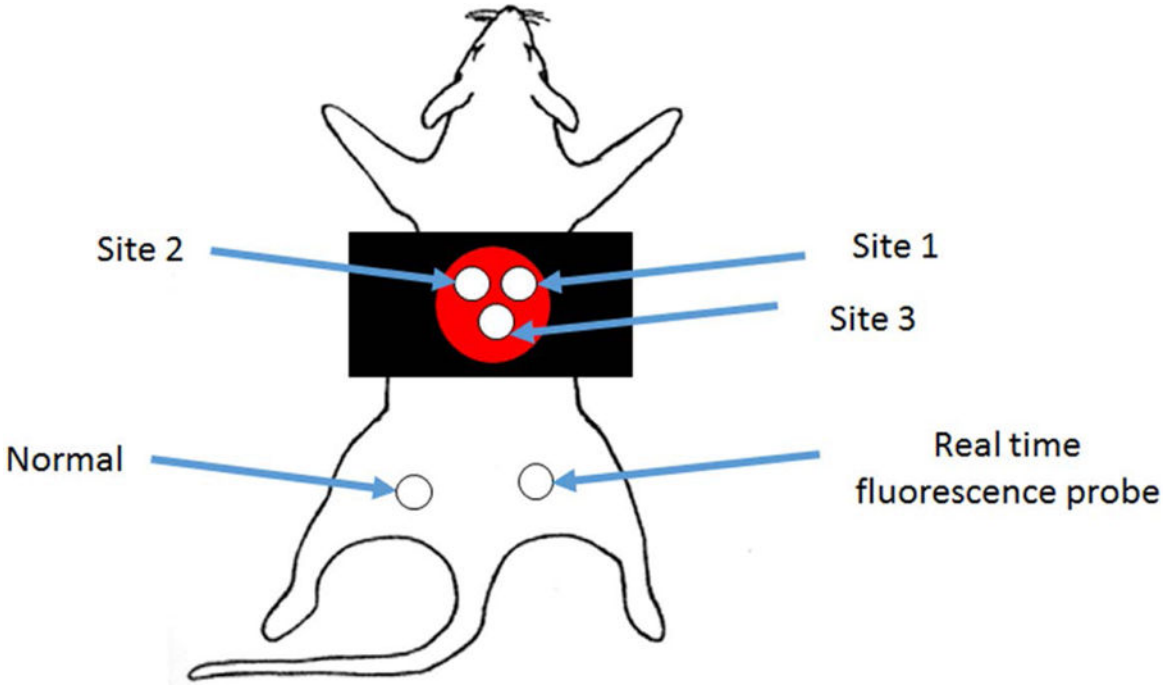


**Figure 1.**

Schematic diagram of mask applied to dorsal side of each subject. Sites represent where treatment occurred and where biopsies were taken. A fourth normal site, not exposed to any treatment light, was also biopsied. The real-time fluorescence probe was placed approximately 50mm from the mask, away from the treatment light.

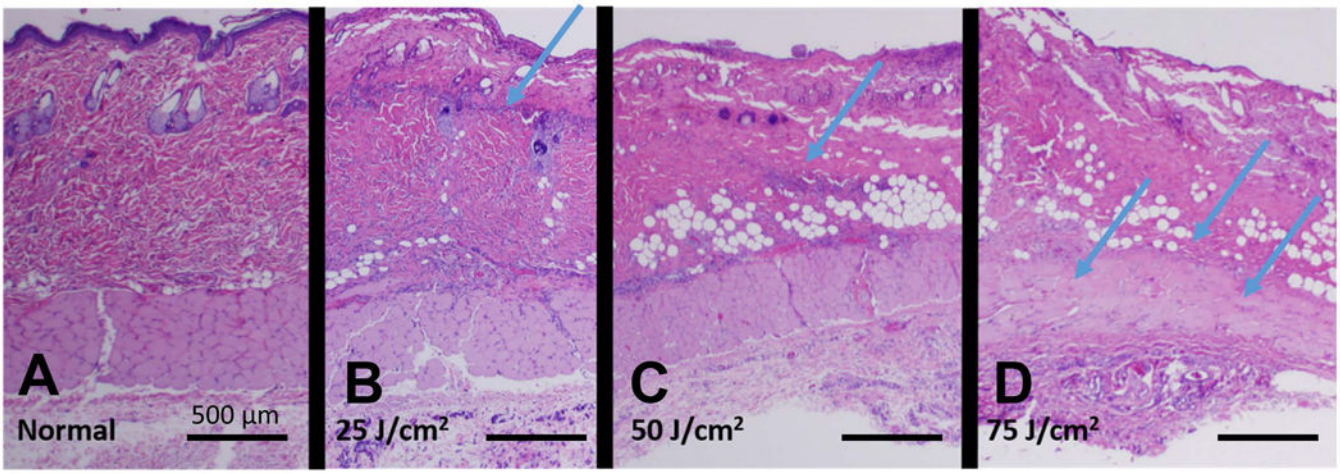


**Figure 2.**

Hematoxylin and Eosin-stained slides of normal, and TS-mediated PDT treated rat skin at 25, 50 and 75 J/cm$^2$ light doses. A) Normal rat skin biopsy taken from site not exposed to light during PDT treatment. B) At 25 J/cm$^2$, a distinct line of neutrophils (arrows) indicates the depth of necrosis, measured at 0.56mm. Vascular changes were observed in the superficial dermis, with minimal epidermal erosion. C) At 50 J/cm$^2$, the depth of tissue necrosis was 0.56mm, a direct result from ischemic necrosis caused by vascular damage. Changes in vasculature were noted below the superficial dermis, in the form of extravasated red blood cells and thrombus formation. D) At 75 J/cm$^2$, ulceration and necrosis was noted from the epidermis through the stratum carnosus. Affected striated muscle was observed to have no nuclei, and was adjacent to healthy striated muscle with nuclei present. Vessels observed were severely damaged, with fibroblast and macrophage proliferation (arrows) noted in damaged tissue areas. Scale bar = 500µm.

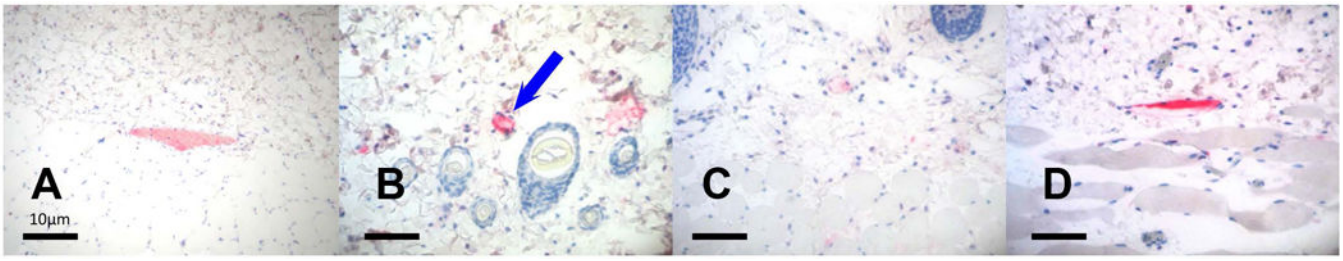


**Figure 3.**

Caspase 3-stained slides of normal, 25 and 75 J/cm$^2$ on Day 0, 9 hour time point. A) Normal skin showing non-specific staining of blood vessel at 140× magnification. B) At 25 J/cm$^2$, specific staining of vessel showing endothelial cell damage at 70× magnification. A properly stained blood vessel is observed in the middle of the image (arrow), and indicates that the endothelial cells were undergoing apoptosis. C) At 25 J/cm$^2$, non-specific staining of blood vessel above layer of adipocytes at 140× magnification. Stain was faint in color and highlighted non-cellular structures and the lumen of the vessel. D) At 75 J/cm$^2$, Caspase 3-specific staining of deep vessels noted above the adipocyte layer. Stain was assigned a score of 3 (dark red color), indicating that the endothelial cells were undergoing apoptosis. Scale bar = 10μm.

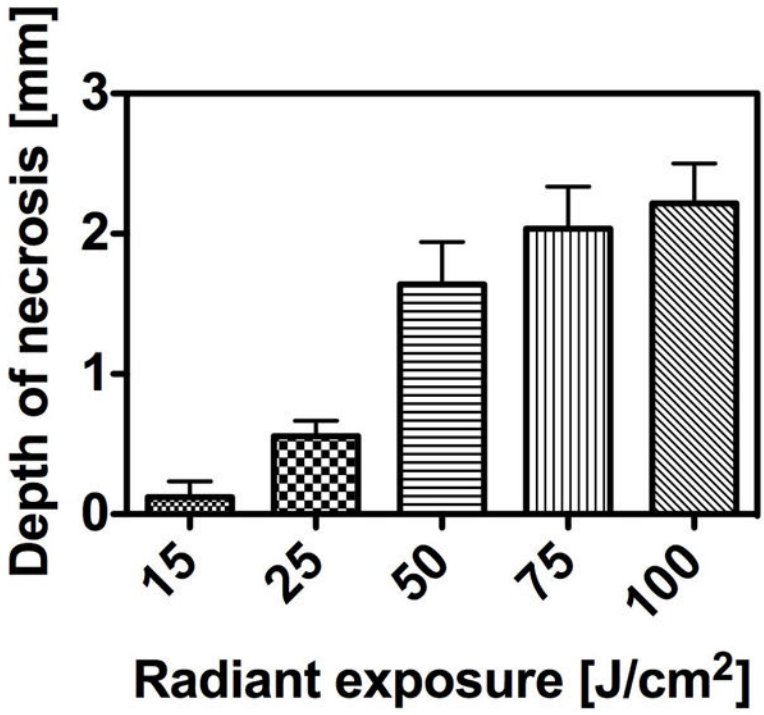


**Figure 4.**

Average depth of necrosis observed at each radiant exposure. A minimum of five depth measurements was measured from each biopsy at each radiant exposure, and a total of 19 observations were made. Error bars represent the standard deviation of the average depth of necrosis.