Use of hyperosmotic chemical agent to improve the laser treatment of cutaneous vascular lesions

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Abstract. A method is presented for decreasing radiant exposures required for photocoagulation of cutaneous blood vessels using a hyperosmotic agent applied to skin prior to laser irradiation. The 50% probability for a given radiant exposure (RE50) to result in photocoagulation of vessels classified by type (arteriole, venule) and diameter was determined following direct (subcutaneous) laser irradiation of 84 vessels in a dorsal skin preparation pretreated with glycerol. Values were compared against results without glycerol pretreatment. A second set of experiments involved irradiation of blood vessels through the skin from the epidermal surface after application of glycerol. Subcutaneous RE50s for vessels treated with glycerol were typically several factors lower than untreated vessels. For example, arterioles in the 80- to 110-μm-diam range in untreated skin had RE50 values $\sim$12 J/cm^2, compared to $\sim$2 J/cm^2 in glycerol-treated cases. Results from epidermal irradiations also indicate that pretreatment with glycerol decreases radiant exposures required for photocoagulation. Vessels were successfully coagulated from the epidermal side in glycerol-treated samples using radiant exposures ranging from 1.6 J/cm^2 to 5 J/cm^2, compared to the 12 to $>$16 J/cm^2 range for control cases. We believe that this method could be a powerful technique for reducing the radiant exposures required for vessel photocoagulation. [DOI: 10.1117/1.2907327]

Keywords: photocoagulation; threshold radiant exposure; optical clearing; optical coherence tomography (OCT); glycerol; hyperosmotic.

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1 Background

1.1 *Cutaneous Vascular Lesions and the Need for Improved Light Penetration*

Cutaneous vascular lesions can be classified into two main categories. The first includes benign disorders, such as the hemangioma (a benign tumor consisting of a dense mass of blood vessels) and the cutaneous nevus (a congenital discoloration of a skin area, also called a strawberry mark or stork mark).[1,2] The second category is made up of vascular malformations, where the lesion is caused by abnormal blood vessel pathology.[1] Included in this class of cutaneous vascular lesions is the port wine stain (PWS), a congenital lesion in which ectatic capillaries make the area appear a dark red color and produce raised nodules protruding above the normal skin surface. Another vascular malformation is leg telangiectasia, small, localized clusters of blood vessels sometimes found deep (mm) below the surface. Telangiectasis can also occur as an extensive network that is much more widespread.[3]

Some of these lesions, such as the PWS, can be psychologically distressing for a patient.[2,4,5] When lesions are located near joints, lips, or the eyes, they can also interfere with normal function and lead to problems such as hypertrophy of skeletal tissue.[1] Because the lesions become increasingly hypertrophic with time, early treatment is preferred.

The only accepted treatment for these vascular lesions is pulsed laser radiation at selected wavelengths that target high-absorption regions of hemoglobin. The process is referred to as selective photothermolysis. Blood vessels are damaged by the increase in temperature resulting from absorption of pulsed laser light by hemoglobin. The objective is to permanently destroy the blood vessels comprising a cutaneous lesion, while sparing surrounding tissue. A number of shortcomings exist in current clinical treatments due to the lack of parameter optimization and sufficient delivery of light to deep-lying blood vessels. Because treatment parameters governing the effectiveness of laser treatment vary greatly from patient to patient, many instances of incomplete destruction of abnormal vessels and clearing of the lesion occur. A typical regimen for PWS treatment requires a series of treatments with a pulsed dye or frequency-doubled laser at intervals of approximately 8 weeks over a period of months. Up to 10 treatments may be required to significantly lighten a birthmark, depending on the size and severity of the lesion. Radiant exposures (REs) are typically in the range of 8 to 12 J/cm^2.

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A limiting factor in the laser treatment of cutaneous vascular lesions is attenuation of the incident light with depth. Attenuation of laser light in biological media occurs by absorption and scattering of light by tissue structures. In many cases, targeted deep blood vessels in a lesion are not sufficiently heated by incident light due to competition from absorption and scattering by other tissue constituents. This competition for laser light decreases the fluence rate (W/cm^2) available for photocoagulation of a blood vessel. An additional factor that may affect photocoagulation success is the velocity of blood in a target vessel. Complete flow cessation of blood in vessels before laser irradiation significantly decreases the fluences required to permanently destroy a blood vessel.[6]

1.2 *Optical and Morphological Effect of Glycerol on Blood Vessels*

Several studies have investigated the effect of hyperosmotic chemical agents, including glycerol, on the optical properties of turbid tissue.[7–14] Early studies by Tuchin et al.[7] demonstrated a dramatic increase in the optical transparency of skin and other turbid tissues after treatment with hyperosmotic agents, including glycerol. Quantitative measurements of skin optical properties following treatment with glycerol revealed up to a factor of 4 decrease in light scattering.[8] The *in vivo* effect of glycerol on cutaneous vasculature has also been studied by Doppler optical coherence tomography (OCT).[9] The addition of glycerol to the subcutaneous side of skin in which blood vessels were located 80 to 100 μm from an exposed subcutaneous surface within a dorsal skin preparation resulted in altered blood vessel morphology and flow. Within 20 min, flow in venules (approximately 100 to 400 μm in diameter) ceased following skin treatment with anhydrous glycerol. Over prolonged periods of treatment to glycerol, flow in arterioles ceased as well. An important finding of that study, however, was that the effect of glycerol on vessels was reversible with hydration—when the skin was hydrated using a physiologic saline solution applied to the skin, flow in arterioles and venules returned to physiologic values and remained at those values. Thus, glycerol alone is not sufficient for long-term vessel stasis.

The known optical/morphological effects of glycerol on skin (increase in light transmission) and on blood flow velocity make it a potential candidate for improvement of laser treatment of cutaneous vascular lesions. Increased light penetration due to glycerol and other hyperosmotic agents should provide for better localization of light on deep blood vessels that previously would not have been targeted due to attenuation effects. The temporary decrease in blood flow velocity could allow for lower radiant light exposures to be used during blood vessel photocoagulation. The current study examined whether pretreatment of skin with glycerol could in fact decrease the radiant exposures required for blood vessel photocoagulation.

2 Materials and Methods

2.1 *Animal Model*

Hamsters were anesthetized with a 4:3 mixture of Ketamine (20 mg/mL): xylazine (100 mg/mL) and 0.15 mL/100 g body weight of the solution was delivered IP. The rodent dorsal skin flap window preparation first developed by Papenfuss et al.[15] was used as an *in vivo* animal model to demonstrate the effect of glycerol on photocoagulation of blood vessels. This *in vivo* model allows the simultaneous observation of the epidermal and subcutaneous sides of the skin while maintaining hydration and function. The preparation has been used as an *in vivo* model for optical imaging of blood vessels on the subcutaneous side of skin while maintaining access to the epidermis.[9,16]

The preparation consists of shaving and epilating the entire dorsal area of a hamster. The skin is pulled away from the body along the dorsal midline and sutured to a vertical C-clamp. A circular region 1 cm in diameter is cut from one single thickness of skin, exposing the subcutaneous side of the opposing thickness of skin. An aluminum chamber is sutured to both sides of the skin, and glass is placed over the cutout section to prevent the tissue from dehydrating on the subcutaneous side. During experiments, this glass is removed for improved imaging, and hydration is maintained with application of physiological saline. The glass window is replaced immediately after each experimental procedure.

Blood vessels observed in the skin of the hamster dorsal window preparation are located in the subdermal fat and connective tissue layers approximately 400 to 700 μm beneath the epidermal surface and about 80 to 100 μm from the exposed subcutaneous surface of a 100-g hamster. Arterioles and venules in the dorsal window preparation range between 50 to 500 μm in (lumen) diameter, with the main arteriole and venule approximately 200 to 500 μm in (lumen) diameter.

2.2 *In Vivo Application of Glycerol*

Anhydrous glycerol was applied to the subcutaneous side of the dorsal skin flap window preparation. The subcutaneous side of the window preparation contains a circular well approximately 2 mm deep and 1 cm in diameter placed directly on the exposed subcutaneous skin. A small opening at the bottom allows the flow of glycerol out of the well. This well was filled with glycerol and continually refilled for a total time of 20 min. Any remaining glycerol was removed at the end of 20 min.

2.3 *Subcutaneous Irradiations*

A total of 46 venules and 38 arterioles were irradiated directly from the subcutaneous surface in six window preparations with radiant exposures ranging from 0.3 J/cm^2 to 7.5 J/cm^2. Three to six irradiations were performed on each window preparation, depending on the anatomical structure of the microvasculature. For instance, in the window preparation in Fig. 1, five areas were irradiated (shown by the circles) to target individual arteriole-venule pairs. This protocol follows that of a previous study in which the laser fluence for permanent damage to cutaneous blood vessels was investigated in the hamster dorsal skin flap model.[17]

Irradiated venules ranged in lumen diameter from 50 to 200 μm. Arteriole lumen diameters ranged from 20 to 140 μm. The subcutaneous side of the window preparation was viewed and recorded through a surgical microscope (ELMO CC421E, Elmo USA, Plainview, NY) prior to, dur-

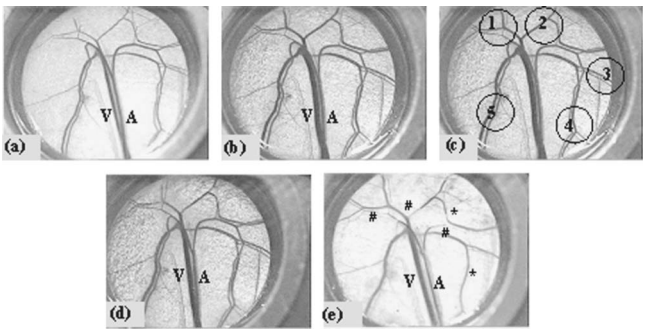

Fig. 1 Subcutaneous side of the hamster dorsal skin flap window preparation. Blood vessels are located approximately 80 to 100 μm from the near surface. (a) Native skin; (b) same window preparation 20 min following pretreatment with glycerol; (c) same image as (b), showing regions of laser irradiation targeting microvessel pairs; (d) window immediately following irradiation of the indicated regions [symbols denote occlusion (*) and focal constriction sites (#)]; and (e) 24 h post-irradiation.

ing, and following irradiation. Blood vessel diameter measurements were made on digital images obtained through the surgical microscope, with the visible vessel diameter corresponding to (lumen) diameter. Vessel diameters in digital images were measured using a line measure tool in NIH Image, a public domain image processing and analysis program (National Institutes of Health, Bethesda, MD). Three measurements were made on the blood vessel in the region of laser radiation and averaged. Each arteriole-venule pair was irradiated a single time with a single pulse from a Versapulse V Vascular (Coherent, Santa Clara, California) laser at 532 nm having a pulse duration of 10 ms and spot size of 3 mm. Following irradiations, the window preparation was hydrated in phosphate buffered saline (PBS). The window preparation was observed 24 h after laser irradiation to assess whether coagulation occurred for each vessel irradiated.

Since previous studies revealed that the fluences required to destroy a blood vessel depended on size and type (arteriole versus venule),[16–18] data analysis for venules and arterioles was done separately, and for each category, vessels were grouped according to inner (lumen) diameter. Venules were categorized in four groups (50 to 80 μm, 80 to 110 μm, 110 to 140 μm, and 140 to 200 μm diameter), and arterioles into three groups (20 to 50 μm, 50 to 80 μm, and 80 to 110 μm). Yes/no (0/1) grading of vessel closure by photocoagulation after 24 h was used to perform probit analysis on the data assuming a sigmoid probability distribution, resulting in 50% probability for threshold radiant exposures (RE50) for vessels of a given size category and type.[17,19] Results were compared to previously published RE50 values determined in the hamster dorsal window preparation,[17] with irradiations and analysis performed in the same manner as the previous study.

2.4 *Epidermal Irradiations*

The effect of glycerol pretreatment on vessel photocoagulation performed from the skin epidermal surface was examined in this set of experiments. Seven dorsal skin flap window preparations were pretreated with glycerol on the subcutaneous side of the window preparation as earlier, and then the main arteriole-venule pair (such as the one labeled *A* and *V* in Fig. 1) was irradiated from the epidermal side to determine the approximate range of radiant exposures required to induce blood vessel stasis in skin treated with glycerol out to 24 h. Four native hamster dorsal skin flap window preparations were irradiated from the epidermal side without pretreatment with glycerol.

The region of skin surrounding the main arteriole-venule pair in each window preparation was first imaged using Doppler optical coherence tomography (DOCT) at 1300 nm on a custom-built system described previously.[20] The skin was imaged from the subcutaneous side, since these blood vessels are located approximately 80 to 100 μm from the subcutaneous surface, allowing for accurate determination of vessel flow and size characteristics, which is often not possible when imaging from the epidermal surface of the window preparation due to the longer optical path. In the case of control samples, the vessels were irradiated from the epidermal side after imaging the native skin using DOCT without pretreating the skin with glycerol. Laser irradiations were achieved by applying a single pulse from a 532-nm, 3-mm spot size, 10-ms pulse duration laser (Versapulse V, Coherent) to the skin without altering its position relative to the DOCT image probe. The skin was imaged before and after irradiation to assess acute changes to blood flow. Physiologic saline was applied to the subcutaneous tissue and the quartz window was replaced in the dorsal window preparation. Samples were then imaged 24 h later to determine whether blood flow remained in the vessels. Vessels in which no blood flow remained at 24 h were judged to be successfully photocoagulated. Visual assessment of coagula in the window preparation was also used to judge photocoagulation. Three of the control samples received a single pulse of 12 or 16 J/cm^2. A fourth control sample received an initial pulse of 4.13 J/cm^2, followed by four increasingly larger pulses up to 16 J/cm^2 when lower radiant exposures failed to result in any change in flow characteristics or the appearance of coagula. The maximum allowable radiant exposure for the laser parameters used was 16 J/cm^2.

In the seven experimental samples, glycerol was applied to the subcutaneous side of the window preparation for 20 min, as described earlier, after first imaging the native skin with DOCT. The glycerol treated skin was then imaged again using DOCT. Laser radiation was applied in the manner described for controls, and the skin was reimaged. Physiologic saline was applied to the subcutaneous side to allow the skin to rehydrate, and the quartz window was reapplied to the dorsal window preparation. Irradiated skin was again imaged after 24 h to judge the presence of vessel stasis (flow cessation of main arteriole or venule after 24 h).

3 Results

3.1 *Subcutaneous Irradiations*

Figure 1 shows images taken of the subcutaneous side of one window preparation prior to treatment with glycerol [Fig. 1(a)], after a 20-min application of glycerol [Fig. 1(b)], after the 20-min glycerol treatment showing specific sites for laser radiation [Fig. 1(c)], immediately after all irradiations had been performed [Fig. 1(d)], and 24 h post-irradiation [Fig. 1(e)]. In this example, several occlusion (*) and focal con-

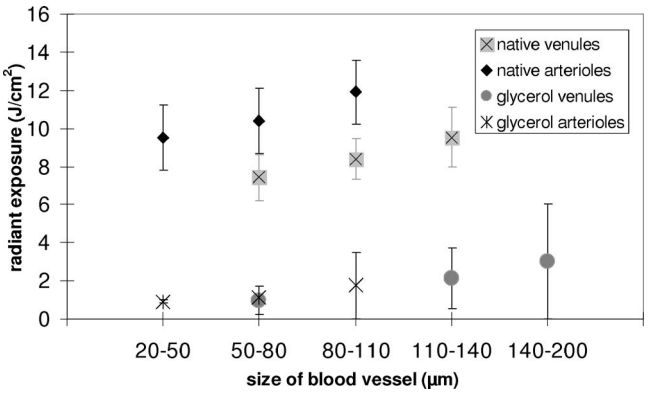

Fig. 2 RE50 values for permanent blood vessel coagulation using a 532-nm laser (10-ms pulse duration, 3-mm spot size) applied to the subcutaneous side of skin in a dorsal window preparation. Native and glycerol pretreated experimental conditions are represented in this plot. Subcutaneous irradiations on native skin are summarized in the native venules and native arterioles data points. Subcutaneous irradiations conducted on skin in which glycerol was applied to the subcutaneous side prior to irradiations are shown by the glycerol venules and glycerol arteriole data points.

striction sites (#) are observed in the microvasculature 24 h post-irradiation. The resulting RE50 values for vessel stasis at 24 h are summarized in Fig. 2 for arterioles and venules in skin treated with glycerol prior to irradiations. Due to the small number of irradiations compared to the number of parameters (vessel size and type), there was insufficient data to estimate the RE50 values for venules 80 to 110 μm in diameter. Results are compared to values from subcutaneous irradiations performed on native dorsal windows not pretreated with glycerol.[17] The radiant exposures required for 50% probability of 24-h stasis are several factors lower in glycerol-treated skin than in native skin (not treated with glycerol prior to laser treatment).

3.2 *Epidermal Irradiations*

Table 1 contains a summary of results for irradiations performed from the epidermal side of the skin. Control samples in which the skin was not pretreated with glycerol prior to laser irradiation are labeled "control." Those in which glycerol treatment occurred prior to laser irradiation are labeled "glycerol." The table specifies the blood vessel diameter according to type (*A* for arteriole, and *V* for venule) as well as the depth of the blood vessel pair from the epidermal surface prior to laser radiation as measured by DOCT. In the treated cases, the first number indicates the depth after glycerol treatment, which was the depth at the time of laser irradiation; the depth before glycerol treatment is specified in parentheses. Depths were estimated using a tissue refractive index of 1.39. The parameters RE_A and RE_V specify the radiant exposure (J/cm^2) applied to the arteriole and venule in each column, followed by a Yes/No column indicating whether the blood vessel was successfully photocoagulated 24 h after laser radiation. A single pulse was applied to each vessel pair, with

Table 1 Epidermal irradiation results shown for individual cases of untreated (control) and glycerol-pretreated window preparations. The table specifies the arteriole (A) and venule (V) lumen diameters, the depth from the epidermal surface at the time of irradiation (in the treated cases, the depth specified is the depth after glycerol treatment when the pulse was applied, with the depth before glycerol treatment specified in parentheses), the RE applied, and whether each vessel was successfully coagulated 24 h after irradiation. A single pulse was applied to each vessel pair with the exception of vessels marked with an [a]. In these three cases (a, h, and i), multiple pulses of increasing RE were applied.

Case	Condition	A diam (μm)	V diam (μm)	Depth from epiderm is	RE_A J/cm^2	24-h coagulation	RE_V J/cm^2	24-h coagulation
a	Control	225	470	710	16[a]	No	16[a]	No
b	Control	—	270	730	—	—	16	Yes
c	Control	200	355	500	16	Yes	16	Yes
d	Control	290	460	410	12	No	12	No
e	Glycerol	220	240	395 (450)	5	No	5	Yes
f	Glycerol	170	215	420 (450)	4.2	Yes	4.2	Yes
g	Glycerol	200	410	471 (655)	4.2	Yes	4.2	Yes
h	Glycerol	300	360	315 (400)	4.9[a]	Yes	1.4	Yes
i	Glycerol	110	260	340 (460)	4.2[a]	Yes	3.1	Yes
j	Glycerol	210	330	495 (565)	1.8	No	1.8	No
k	Glycerol	250	300	300 (375)	1.6	Yes	1.6	Yes

[a]Applied REs (J/cm^2) in cases of multiple pulses: sample a 3.75, 5.0, 6.5, 8.0, 10.0, 12.0, 14.0, 16; sample h 1.4, 4.2, 4.9; sample i: 3.1, 4.2.

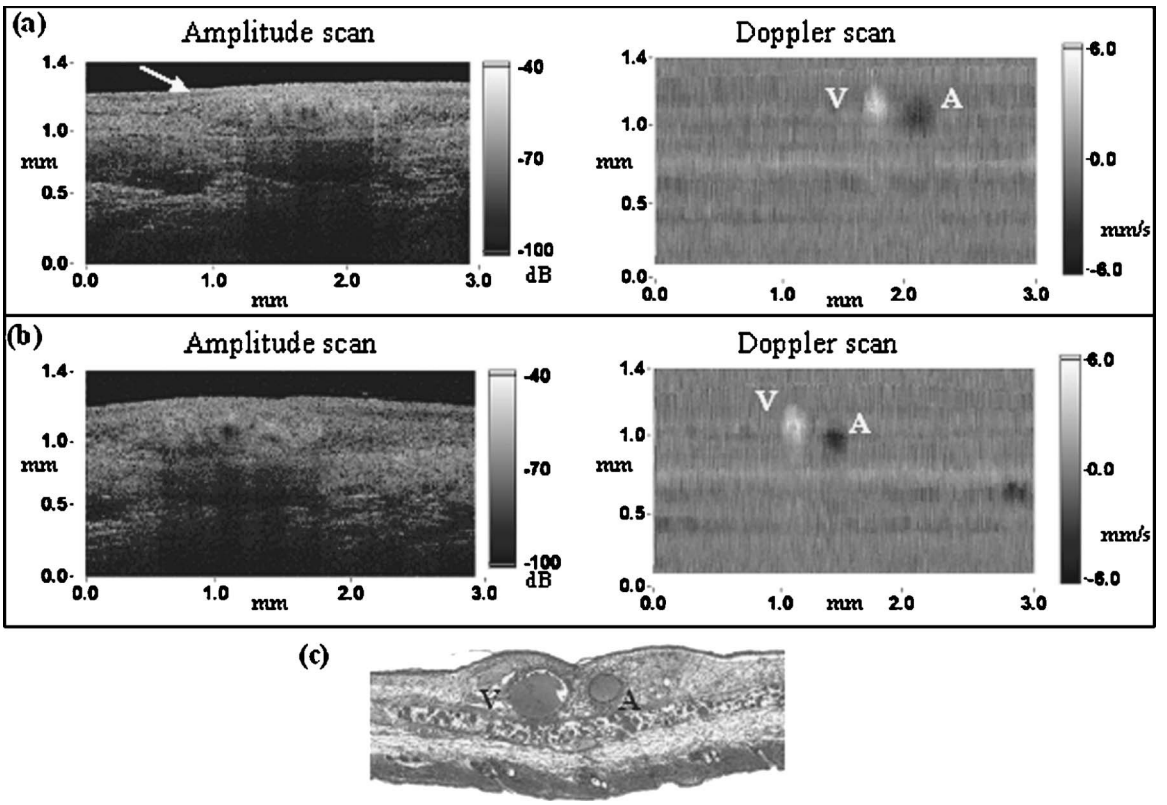

Fig. 3 Amplitude and Doppler images of skin in the hamster dorsal skin flap window preparation. (a) The skin shown is a control sample prior to irradiation. The surface at the top marked by the arrow is the subcutaneous connective tissue. (b) Subcutaneous side of control sample 24 h after irradiation of 16 J/cm^2. Flow in both the arteriole and the venule remains. (c) H&E labeled section of control case (a) 24 h following epidermal irradiation with 16 J/cm^2. Both the arteriole and the venule remain uncoagulated.

the exception of vessels marked with an [a]. In these three cases (a, h, and i), multiple pulses of increasing RE were applied, with the maximal RE applied indicated in the table and individual pulses listed below the table. Application of incrementally higher laser pulses occurred in these three cases, one control case (a) and two experimental cases (h and i). In each of these cases, an initial RE failed to result in any change in blood vessel flow or morphology. A larger RE was applied 1 h later. In the control case a, several REs (listed below the table) applied approximately 1 h apart were unsuccessful at inducing stasis in either the arteriole or venule when assessed for flow 24 h later. In case h, the first RE of 1.4 J/cm^2 immediately stopped flow in the venule but not the arteriole. Two higher REs were applied 1 h apart, with the third stopping flow in the arteriole—flow had not returned 24 h later. Similarly, the first RE applied in case i resulted in venule flow ceasing, but a higher RE applied 1 h later was required to stop flow in the arteriole.

In the case of glycerol pretreated samples, the RE that successfully induced vessel stasis ranged from 1.6 J/cm^2 to approximately 5 J/cm^2. In two of the control cases, $RE > 12$ J/cm^2 were required for vessel stasis (i.e., RE values of 12 or 16 J/cm^2 were not sufficient for coagulation). In the other two control cases, vessel flow ceased by using 16 J/cm^2.

Representative DOCT images from a control case and an experimental case irradiated from the epidermal side follow in Figs. 3 and 4. Figure 3 shows amplitude and Doppler DOCT scans of a control case. Figure 3(a) shows native hamster skin in the dorsal window preparation. A cross section of the skin is shown, with the air-subcutaneous surface shown in the top part of the image (arrow). The amplitude image represents the intensity of backscattered light from the tissue. The Doppler image represents the frequency-shifted light from scatterers in the skin, namely, flowing red blood cells. One cannot specifically identify blood vessels in the amplitude image. (Vertical shadowing beneath blood vessels indicative of increased attenuation through the blood relative to surrounding tissue suggests that blood vessels may be in the skin, but does not definitely identify blood vessel location.) However, specific localization of an arteriole and a venule is possible in the Doppler image due to the Doppler shift.

Figure 3(b) shows the same venule-arteriole pair 24 h post-irradiation with 16 J/cm^2. It is evident from the Doppler image in Fig. 3(b) that flow remains strong in both blood vessels despite application of the maximal allowable RE for the laser settings used. Following imaging, a biopsy was obtained of the imaged skin region and processed for histology. A hematoxylin and eosin (H&E) labeled section is shown in Fig. 3(c). Both blood vessels remain uncoagulated.

Figure 4(a) shows an area of skin in the window preparation containing a venule-arteriole pair prior to treatment with glycerol and irradiation. Figure 4(b) shows the same venule-arteriole pair after 24 h. In this case, the location of the arte-

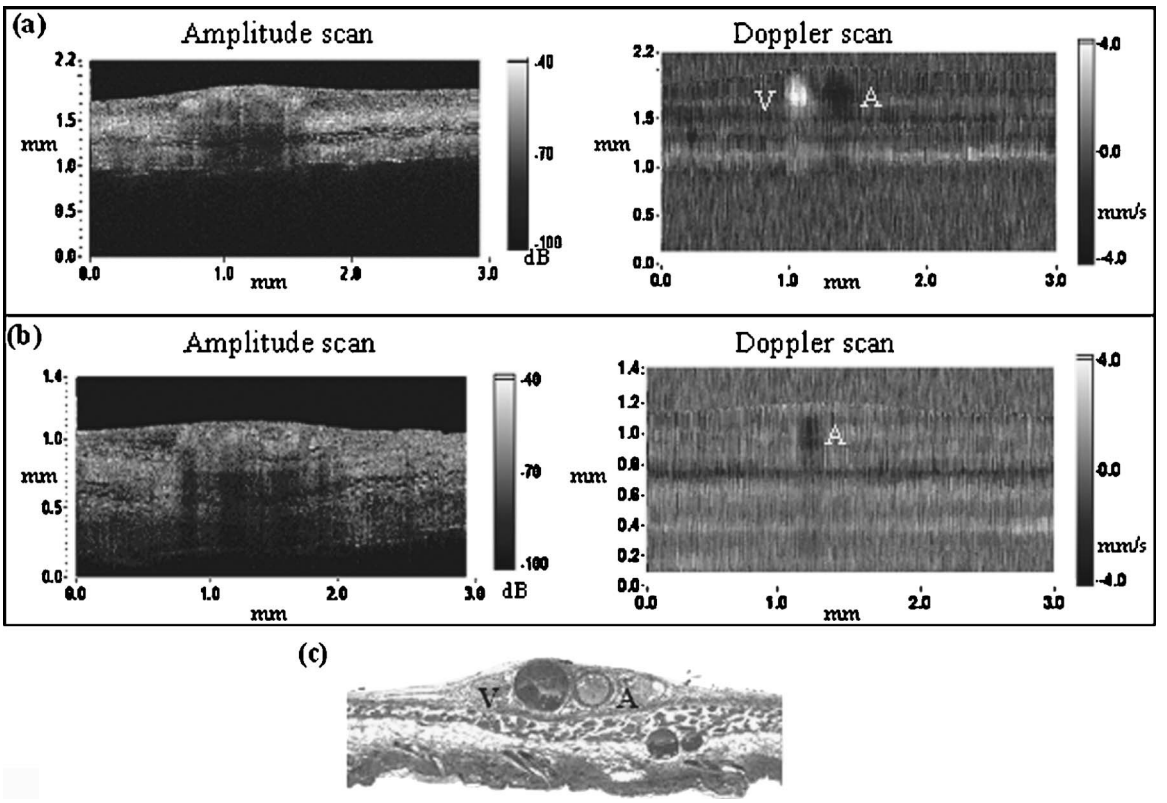

Fig. 4 (a) Amplitude and Doppler images from native experimental dorsal skin flap before application of glycerol or laser radiation. (b) Amplitude and Doppler images from dorsal skin flap 24 h after laser radiation (4.2 J/cm^2) applied to the epidermal side of the skin following a 20-min application of glycerol to the subcutaneous tissue. The arteriole and venule are identified in the amplitude image; however, no flow remains, as can be seen from the lack of Doppler shifts in the Doppler image. (c) H&E stained section 24 h after glycerol pretreatment and irradiation.

riole and venule is suggested by morphology and shadowing in the amplitude scan; however, the Doppler scan of Fig. 4(b) shows no flow remaining in the venule but flow remaining in the arteriole. The corresponding H&E stained histology of Fig. 4(c) confirms the successful coagulation of the venule in this example, with a clear coagulum present in the venule. The arteriole appears normal in this H&E section. Two smaller blood vessels present in the histological section are not obvious in the OCT images, perhaps due to light attenuation preventing a strong Doppler signal from being detected in these vessels, which are deeper than the targeted vessels, or perhaps because the section is displaced laterally from the imaged plane. Nonetheless, the imaged blood vessels are captured in the histological section, confirming the DOCT results.

4 Discussion

4.1 *Effect of Glycerol on Skin and Cutaneous Blood Vessels*

The purpose of the current study was to evaluate the potential use of the optical clearing agent glycerol as a candidate for reducing the radiant exposures required for vessel photocoagulation. Vessels were examined 24 h following laser radiation. Glycerol has been shown in several studies to increase the transmission of visible and near-infrared light into normally turbid tissues, including skin. Specifically, the agent leads to a decrease in light scattering, which increases the penetration depth of light considerably. Current hypotheses suggest that the effect may be partially a consequence of local tissue dehydration leading to improved index matching between tissue constituents[13] and/or reversible dissociation of collagen fibers that may reduce light scattering.[10,21] Both hypotheses indicate that collagen may be a main contributor to the optical clearing effect, although the role of the cells has not been closely investigated.

A potential advantage of increased light penetration for clinical applications of lasers is that better targeting of light to a subsurface target may be achieved. For example, a collimated or focused beam may be maintained deeper in tissue, and blood vessels that may not have been previously coagulated because of the inability of light to reach them may be more specifically targeted. In some cases, however, it is possible that a decrease in light scattering could in fact increase the radiant exposures required for vessel stasis, since some photons contributing to photocoagulation may include those scattered into blood vessels from the periphery.[22]

A second interesting effect of glycerol on tissue is that in addition to altering the bulk optical properties, this hyperosmotic agent also alters the flow characteristics of blood vessels.[9,23–25] Treatment of tissue with glycerol has been shown to induce a reduction in blood flow in these studies. This effect was demonstrated in a previous study in the hamster dorsal skin flap, in which treatment of the dorsal skin flap with glycerol led to a reversible decrease in blood flow of measured microvasculature.[9] Application of glycerol to the subcutaneous side of the skin flap preparation resulted in

venule stasis, with arteriole stasis occurring over longer application times. Measurements were made using DOCT at 1300 nm. With cessation of blood flow following glycerol treatment, the intensity of light backscattered from within the lumen decreased substantially, which allowed for improved contrast in images of skin containing blood vessels because (1) the blood vessel could be visualized in intensity scans without Doppler imaging and (2) the characteristic shadowing effect seen below blood vessels at this wavelength was eliminated, allowing the rest of the dermis to be visualized and indicating decreased light attenuation. Although glycerol did lead to vessel stasis in the short term of 20 min, upon rehydration with physiologic saline, blood flow returned to physiological values in all studied vessels and remained. This is an important distinction from other studies reporting a decrease in blood flow by glycerol, where rehydration of the tissue was not specifically aided with the addition of saline, as in Vargas et al.[9] In the current study, all samples were hydrated with saline to eliminate the possibility that glycerol treatment alone induced vessel stasis at 24 h. Given the results of Vargas et al.,[9] we believe that the 24-h vessel stasis effect reported in the current study is not due to pretreatment of glycerol alone—but rather is due to laser radiation coagulating blood vessels—this is specifically confirmed by histology. Glycerol simply creates the temporary optical and flow characteristics that aid in the photocoagulation of vessels. Effects that are attributable to glycerol alone are reversed by hydration with saline.

It is noted that glycerol treatment leads a decrease in thickness in samples. In the current study, the average decrease in distance from the epidermal surface to targeted blood vessels was $18.3 \pm 8.1\%$, determined from the preglycerol and postglycerol distances measured in OCT scans and indicated in Table 1. Determination of true physical distance changes requires knowing the change in refractive index, which is unknown in these studies. We have assumed an unchanging refractive index of 1.39. It is possible that a change in tissue refractive index occurs with glycerol treatment—although it is not known how much of a change occurs, one can surmise a small increase will occur if either glycerol enters the tissue or water is removed, making 18.3% a conservative estimate. Glycerol refractive index at its maximal (anhydrous) is 1.47, also the value for the highest refractive index component of skin, collagen, in its dehydrated form.[26] If one assumed a final refractive index of 1.47, the maximal depth change is $22.7 \pm 7.9\%$. This is likely an overestimation, since it is probable that an intermediate value between the native tissue value, 1.39, and the value for glycerol and dry collagen, 1.47, is reached.[23] Rehydration overnight following laser irradiations restored thicknesses to baseline values. This change in thickness is not likely a factor in the subcutaneous irradiation study, as vessels are already located at/near the irradiated surface.

4.2 *Subcutaneous Versus Epidermal Irradiations*

Pretreatment with glycerol subjects skin to changes that reduce the required radiant exposures for photocoagulation at 24 h. The subcutaneous irradiation study in which vessels were directly targeted from the subcutaneous side demonstrates a reduction in RE50 due to direct effects of glycerol on the vessel, possibly flow, since vessels were located at/near the irradiated surface. OCT amplitude scans revealed that treatment with glycerol leads to a reduction in light attenuation through the blood vessels,[9] and additional studies reported a higher light transmittance through blood and lowered attenuation coefficient after mixing with glycerol.[11,24,27] These indicate that it is unlikely that the effect that leads to reduced RE50 in subcutaneous irradiations is a direct optical effect on blood.

In the case of epidermal irradiations, optical and physical effects of glycerol on the skin become a factor, since light must now travel through the epidermis and dermis to reach the targeted subcutaneous blood vessels. There is an 18% decrease in depth, which may be considered to partially be due to a decrease in physical depth and partially to a decrease in optical depth following an increase in light transparency of the tissue due to an up to fourfold decrease in scattering. While we did not note the up to tenfold decrease in RE observed for direct subcutaneous irradiation of vessels, our preliminary results for epidermal irradiation indicate a significant decrease in RE. Native (control) REs ranged from 12 to $>16\ \mathrm{J/cm^2}$, whereas glycerol reduced REs to 1.6 to 5 $\mathrm{J/cm^2}$. Although the study involving epidermal irradiations of glycerol treated skin is preliminary, the results indicate with reasonable certainty that the optical clearing technique will allow use of lower laser fluences when treating cutaneous blood vessel lesions. Further study will differentiate the effect of reduced scattering on the fluences required to destroy a vessel from the effect of flow changes and examine optical and physical path length changes.

5 Conclusion

The presented data offers experimental support for using glycerol and potentially other optical clearing agents to enhance the laser treatment of cutaneous vascular lesions by significantly reducing the RE required for permanent vessel photocoagulation. Vessels were examined 24 h following laser radiation with or without glycerol pretreatment in this study. While this time point has previously been accepted as an appropriate time to judge "permanent" vessel damage, recent evidence suggests that a time point of 7 days may be required to determine whether irreversible damage has occurred by laser radiation.[28] Nonetheless, the current results indicate that a combined glycerol/laser treatment may be effective for reducing radiant exposures required for laser photocoagulation of cutaneous blood vessels.

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