

Photocoagulation of Dermal Blood Vessels With Multiple Laser Pulses in an In Vivo Microvascular Model

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Background/Objectives: Current laser therapy of port wine stain (PWS) birthmarks with a single laser pulse (SLP) does not produce complete lesion removal in the majority of patients. To improve PWS therapeutic efficacy, we evaluated the performance of an approach based on multiple laser pulses (MLP) to enhance blood vessel photocoagulation.

Study Design: The hamster dorsal window chamber model was used. Radiant exposure (RE), pulse repetition rate (f_r), total number of pulses (n_p), and length of vessel irradiated were varied. Blood vessels in the window were irradiated with either SLP with RE of 4–7 J/cm² or MLP with RE per pulse of 1.4–5.0 J/cm², f_r of 0.5–26.0 Hz, and n_p of 2–5. The laser wavelength was 532 nm and pulse duration was 1 ms. Either a 2 mm vessel segment or entire vessel branch was irradiated. Digital photographs and laser speckle images of the window were recorded before and at specific time points after laser irradiation to monitor laser-induced blood vessel structural and functional changes, respectively.

Results: We found that: (1) for a SLP approach, the RE required to induce blood vessel photocoagulation was 7 J/cm² as compared to only 2 J/cm² per pulse for the MLP approach; (2) for MLP, two pulses at a repetition rate of 5 Hz and a RE of 3 J/cm² can induce photocoagulation of more than 80% of irradiated blood vessel; and (3) irradiation of a longer segment of blood vessel resulted in lower reperfusion rate.

Conclusions: The MLP approach can induce blood vessel photocoagulation at much lower RE per pulse as compared to SLP. The 5 Hz f_r and the need for two pulses are achievable with modern laser technology, which makes the MLP approach practical in the clinical management of PWS birthmarks. *Lasers Surg. Med.* 44:144–151, 2012. © 2012 Wiley Periodicals, Inc.

Key words: laser speckle imaging; dorsal window chamber; laser dermatologic surgery; vascular malformation; port wine stain; angiogenesis

INTRODUCTION

Port wine stain birthmarks (PWS) are congenital, progressive vascular malformations of human skin with an incidence rate of 3 per 1,000 live births [1,2]. PWS are characterized by an increase in blood vessel size and a decrease in perivascular nerve density [3–5]. The current treatment of choice for PWS is the pulsed dye laser (PDL) [6,7] with dynamic skin cooling [8,9]. However, complete PWS blanching is rarely achieved for many patients even after numerous PDL treatments [10–12]. Many factors contribute to incomplete PWS blanching, including the presence of large blood vessels which can only be partially photocoagulated by PDL due to high superficial light absorption by hemoglobin [13–15], epidermal melanin that reduces light delivery to targeted PWS vessels, and regeneration of photocoagulated blood vessels due to angiogenesis [16–19].

The multiple laser pulses (MLP) approach has been successfully used to treat facial and leg telangiectasia [20–22]. Histological evaluation of laser-irradiated normal and PWS skin revealed that MLP induced coagulation of deeper vasculature as compared to the single laser pulse (SLP) approach [23,24]. The introduction of multiple cryogen spurts applied intermittently with MLP makes this approach even more appealing because the epidermis can be effectively cooled between the consecutive laser pulses, thus enabling safe delivery of higher total light dosages [15,25,26]. From a thermophysical point of view,

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the rationale of the MLP approach is that the delivery of subsequent pulses before the blood vessels cool to the baseline temperature can increase the temperature of blood vessels to a greater degree as compared to a SLP [15,25]. From a biological point of view, researchers proposed that MLP induce summation of irreversible, thermal injury from a series of lower-peak temperature heating cycles [27]. The different views on the MLP approach explain why the inter-pulse interval varied from 0.25 seconds to 30 minutes and the total number of pulses (n_p) varied from 2 to 81 in previous studies of the MLP approach [15,20–26]. We believe that the large variation in the treatment parameters hinders the practical application of the MLP approach. To address this issue, we used an *in vivo* microvascular model to elucidate the mechanism of MLP-induced photocoagulation. The objective of the present study is to investigate the effects of radiant exposure (RE), pulse repetition rate (f_r), n_p , and irradiated vessel length on the short-term photocoagulation and long-term removal of blood vessels using the MLP approach as compared to SLP.

STUDY DESIGN/MATERIALS AND METHODS

In Vivo Animal Model

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee, University of California, Irvine. Adult male Golden Syrian hamsters (90–120 g) were used in this study. A dorsal window chamber (DWC) was installed on each animal. This model, first described by Algire [28], consists of a lengthwise fold of dorsal skin with an implanted clear glass window that permits *in vivo* visualization and irradiation of the subdermal blood vessels. The window chamber, when properly prepared, provides excellent viewing of subdermal blood vessels up to 4 weeks [17,29]. Details of the chamber structure and surgical procedure can be found elsewhere [30–32]. Briefly, after the animal was anesthetized with a cocktail of ketamine/xylazine, the dorsal skin was shaved, epilated, and lifted to form a skinfold. A pair of titanium window frames was attached to the front and back sides of the dorsal skinfold with screws and sutures. One layer of skin and subcutis with the panniculus carnosus was completely removed within the circular area of the frame's observation window to expose the subdermal blood vessels in the underlying intact skin. A thin glass window (12 mm diameter, 0.2 mm thickness) was then inserted into the window frame to protect the subdermis from dehydration and contamination. The window frames were strategically placed on the backs of the animals to enable visualization of a tree-like vascular network for the experiments.

Laser Irradiation

Laser irradiation was performed on the window glass (subdermal) side of the preparation. Two millimeter blood vessel segments or the entire vessel branches were irradiated with a frequency-doubled Nd:YAG laser (Dualis VP+, Fotona, Ljubljana, Slovenia) which emits single or

multiple pulses at a wavelength of 532 nm. The duration of an individual pulse is 1 ms, and the RE could be varied from 1.4 to 7 J/cm² with the 2 mm spot used in this study. For MLP, the n_p could be varied from 2 to 5 and the f_r from 0.5 to 26 Hz. Laser pulse energies were verified using an energy meter (FL250A-SH with Nova display, Ophir, Logan, UT).

Color and Laser Speckle Imaging

Digital color photos and laser speckle images (LSI) of the windows were acquired prior to, shortly after laser irradiation, and daily thereafter for 2 weeks. After the day of window implantation, hamsters were anesthetized with a mixture of oxygen and isoflurane (3%) through a nosecone. Color images of the subdermal sides of windows were taken while white light illumination was delivered to the subdermal side or through the epidermal side of the window. Although color images can document blood vessel structural changes after laser irradiation, they cannot be reliably used to judge whether blood flow has completely stopped. Therefore, LSI was used to determine blood flow dynamics in the window [17,33–35]. During LSI, the window was trans-illuminated with a CW HeNe laser to produce a speckle pattern. When blood flow is present, the speckle pattern varies with time; otherwise, the pattern is static. The speckle patterns were integrated over 10 ms with a CCD camera and processed with a sliding-window-based algorithm [36–38] to visualize blood flow in the window.

Data Analysis

We only analyzed the response of venules to laser irradiation, though arterioles and venules in the DWC usually run in pairs, because PWS are malformations of capillaries and/or post-capillary venules. We first identified blood vessels that were completely photocoagulated by comparing color images and LSI flow maps recorded before and after laser irradiation. The criterion was that flow was completely stopped in the photocoagulated vessels. The coagulated blood vessels were then tracked in the color images and flow maps in the following 2 weeks to determine whether reperfusion occurred.

RESULTS

Effect of Radiant Exposure on Blood Vessel Photocoagulation

Experiments were performed first to determine the threshold RE per pulse to coagulate 100–250 μ m diameter blood vessels in the DWC model for both SLP and MLP. Results are shown in Figure 1. For SLP, a RE of 7 J/cm² was required to induce photocoagulation in most (i.e., 8 out of 9) irradiated blood vessels. In contrast, MLP with five pulses at $f_r = 26$ Hz could induce photocoagulation in practically all irradiated blood vessels at RE = 2 J/cm² and higher. Fitting the Boltzmann's dose-response function to the observed coagulation percentage as a function of RE yields the 50% probability RE value of

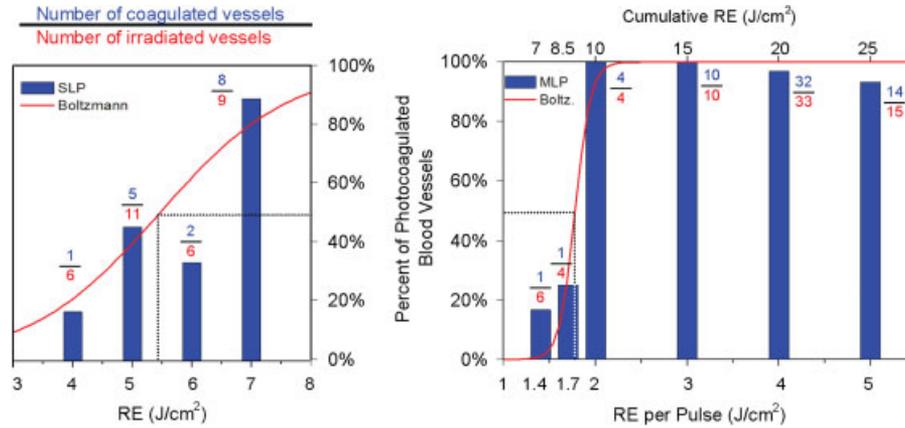


Fig. 1. Effect of radiant exposure (RE) on blood vessel photocoagulation. The red curves are the Boltzmann's dose-response function fitted to the observed photocoagulation percentage as a function of RE. The numbers next to the columns are number of coagulated vessels versus number of irradiated vessels. SLP, single laser pulse; MLP, multiple laser pulses. For MLP, the total number of pulses was 5 and pulse repetition rate (f_r) was 26 Hz.

1.78 J/cm² for the MLP approach, obviously very different from the value of 5.45 J/cm² obtained for the case of SLP.

Effect of Total Number of Pulses on Blood Vessel Photocoagulation

As shown in Figure 1, the threshold n_p was not more than 5 when RE was 2 J/cm². Further experiments were conducted using the DWC model to determine the threshold n_p when RE was 3–5 J/cm² and f_r was 10 Hz. A total of 9 animals, 3 for each RE, were used in this study group. One example, when RE was 3 J/cm², is shown in Figure 2. Figure 2a shows an image of the DWC before laser irradiation and Figure 2c is the LSI flow map. Vessel diameters, which ranged from 117 to 218 μ m, are also shown in Figure 2a. The yellow circles mark the sites of laser irradiation, which have a diameter of 2 mm. Figure 2b and d shows a color image and a LSI flow map of the DWC, respectively, after laser irradiation. When n_p was 2 or higher, a thermal coagulum (dark red color) [39–41] could be seen in the irradiated sites in Figure 2b, and no flow was detected in Figure 2d which indicates complete blood vessel coagulation. A summary of the results is shown in Table 1. It can be seen that more than 80% of the irradiated blood vessels were coagulated when n_p was 2 or higher.

Effect of Pulse Repetition Rate on Blood Vessel Photocoagulation

Results in Figure 1 and Table 1 confirm that effective blood vessel photocoagulation can be achieved when f_r was 26 and 10 Hz. More experiments were conducted to determine if blood vessels could be coagulated with a lower f_r at a RE per pulse of 4 J/cm² and a n_p of 2. A total of 4 animals were used in this study group. Figure 3 shows an example which demonstrates the effect of f_r on photocoagulation of blood vessels in the DWC model. Color image and LSI flow map of the DWC before laser irradiation are

shown in Figure 3a and c, and the same DWC after laser irradiation is shown in Figure 3b and d. Three f_r values were used, 0.5, 1, and 5 Hz. Figure 3d shows that blood flow was clearly detected in the blood vessels irradiated at a f_r of 0.5 or 1 Hz. Alternatively, blood flow was completely stopped in the blood vessels irradiated at a f_r of 5 Hz. It can be noted that a blood coagulum was present downstream of the irradiation site for a blood vessel irradiated at a f_r of 0.5 Hz (arrows in Fig. 3b and d). This could imply that the coagulum was not large enough to block the entire vessel and was washed away from the irradiation site. A summary of the results is shown in Table 2. When f_r was 1 Hz or lower, only 1 out of 8 irradiated blood vessels were coagulated. In contrast, 5 out of 6 irradiated blood vessels were coagulated when f_r was 5 Hz.

Effect of Irradiated Vessel Length on Long-Term Blood Vessel Removal

A 2 mm segment of blood vessels was irradiated in the above animal experiments, although irradiation of a longer segment would be possible by using a larger laser spot size. However, unlike our DWC model, where blood vessels run perpendicularly to the laser beam axis, malformed post-capillary venules in PWS dermis may run nearly parallel with the laser beam, from papillary loops to the superficial horizontal plexus. As a result, coagulation of a long segment of a PWS blood vessel with a SLP is unlikely due to the limited PDL penetration depth in human skin. The MLP approach is capable of damaging vasculature located deeper in the skin [15,26], and thus, a longer vessel segment might be coagulated as compared to SLP. It is therefore of interest to study the effect of irradiated vessel length on long-term blood vessel removal.

In contrast with the first part of this study, where only isolated 2 mm vessel segments were irradiated (see Figs. 2a and 3a), we irradiated entire vessel branches

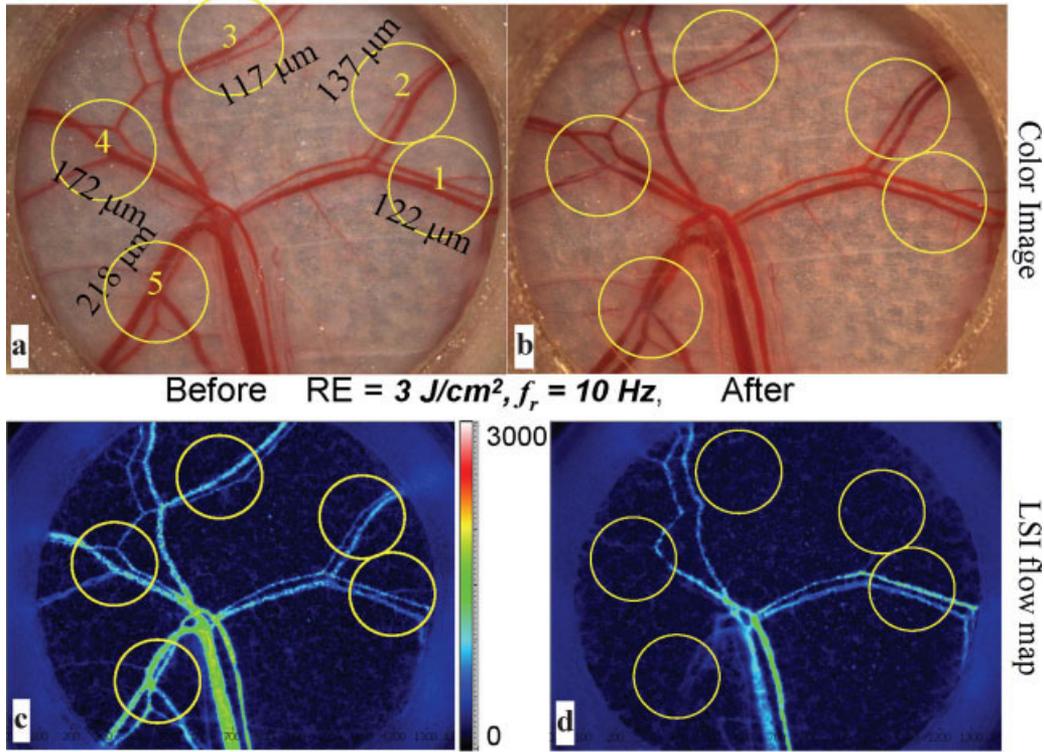


Fig. 2. Effect of total number of pulses (numbers in the circles) on blood vessel photocoagulation. **a,b**: Color images of the subdermal side of the window before and after laser irradiation. **c,d**: LSI flow maps. Blood vessels were completely coagulated when the total number of pulses was 2 or higher. Radiant exposure (RE) was 3 J/cm^2 with a pulse repetition rate (f_r) of 10 Hz. The diameters of venules, which are larger and darker, are shown in (a).

(Fig. 4a) with MLP with a RE of 4 J/cm^2 , a n_p of 5 and a f_r of 26 Hz. In an earlier study, it was found that 95% of the coagulated blood vessels reperfused within 2 weeks when isolated 2 mm vessel segments were irradiated at such conditions [42]. When the vessel branches were irradiated as shown in Figure 4, there was no evidence of reperfusion or regeneration of the coagulated vessel branches after 2 weeks in the presented example. From a total of 11 irradiated blood vessel branches in 6 animals treated in

the described manner, only 45% of the coagulated blood vessel branches reperfused within 2 weeks (Table 3).

DISCUSSION

Our animal study indicated that the threshold RE per pulse of 2 J/cm^2 for MLP to induce blood vessel photocoagulation is substantially lower as compared to that of 7 J/cm^2 for SLP (Fig. 1). Therefore, MLP approach may achieve a better therapeutic outcome of PWS in cases where the maximum permissible RE per pulse, limited by the epidermal damage threshold, is insufficient to induce persistent vascular shutdown. The data also indicate that the total quantity of light energy (e.g., cumulative RE in Fig. 1) that can be safely delivered with the MLP approach is considerably higher than with the SLP approach.

The n_p is a unique parameter for a laser based on the MLP approach. The selection of n_p is a balance between efficacy and safety for MLP. If n_p is too low, the largest PWS blood vessels may not be coagulated; if n_p is too high, heat diffusing from the vessels to the perivascular tissue may cause unwanted thermal injury. In theory, vessel diameter will certainly influence the selection of n_p

TABLE 1. Summary of the Effect of Total Number of Pulses (n_p)

n_p	Number of venules		
	Irradiated	Coagulated	Coagulation %
1	8	2	25
2	10	8	80
3	7	7	100
4	8	8	100
5	7	6	86

RE = $3\text{--}5 \text{ J/cm}^2$; $f_r = 10 \text{ Hz}$.

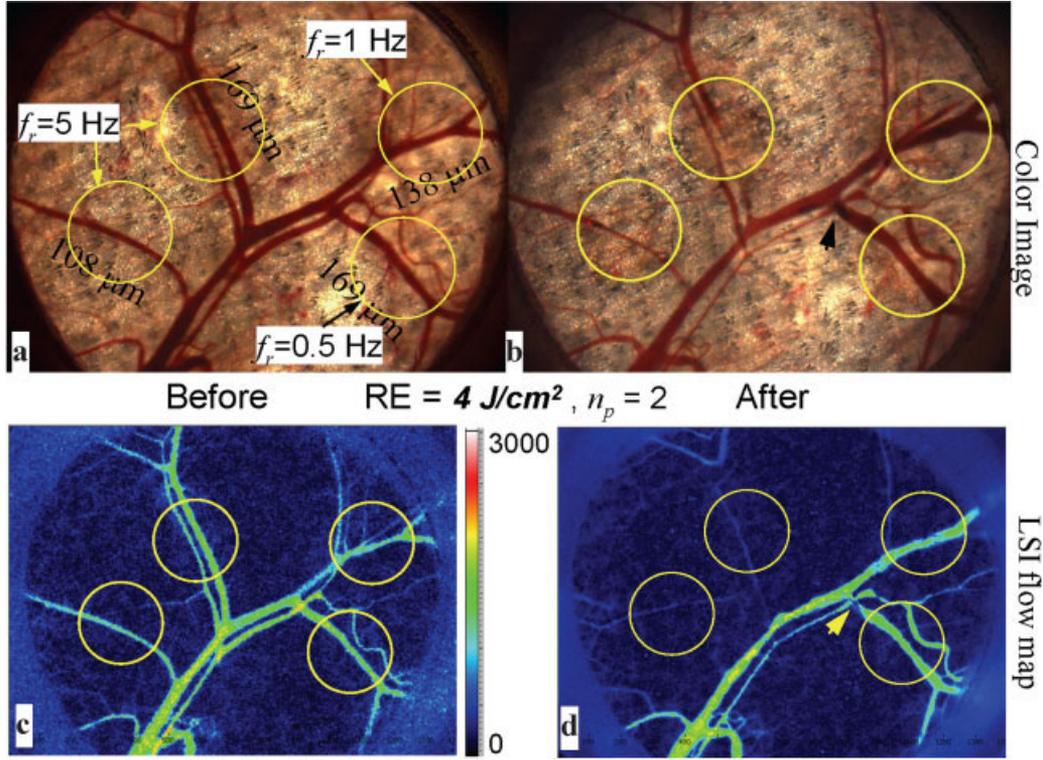


Fig. 3. Effect of pulse repetition rate (f_r) on blood vessel photocoagulation. **a,b**: Color images of the subdermal side of the window before and after laser irradiation. **c,d**: LSI flow maps. Blood vessels were completely coagulated when f_r was 5 Hz. Radiant exposure (RE) was 4 J/cm^2 and total number of pulses (n_p) was 2. The diameters of venules, which are larger and darker, are shown in (a). Arrowheads: blood coagulum.

because heat diffusion dynamics depend on the diameter of the vessel which serves as a heat source. In addition to vessel diameter, RE also affects n_p . Laser pulses with higher RE can heat the blood vessel to a sufficiently high temperature with a lower n_p . As shown in Table 1, more than 80% of the irradiated blood vessels were coagulated when two or more pulses were used in conjunction with a RE per pulse of 3 J/cm^2 or higher. Severe collateral skin damage such as ulcer was not observed in this study.

From a thermophysical point of view, the main criterion to determine f_r is that a significant portion of heat generated by successive laser pulses accumulates in the

targeted PWS blood vessels, and thus, the core intravascular blood vessel temperature increases substantially with each subsequent laser pulse. Another factor influencing the selection of f_r is that the time interval between successive laser pulses must be long enough to allow sufficient cooling of the basal layer of the epidermis, which will depend primarily on the concentration and depth distribution of epidermal melanin. As shown in Table 2, blood vessels could be coagulated when f_r was 5 Hz or higher. The corresponding inter-pulse interval of 200 ms for $f_r = 5 \text{ Hz}$ is much longer than the 80 ms required for typical cryogen spray delivery in clinical practice, which allows for sufficient epidermal cooling and protection.

Our data showed that blood vessels could not be coagulated when f_r was 1 Hz or less. This outcome most likely is due to the excessive vessel temperature decrease that can occur during the 1-second interval between pulses. In the present study, a RE of 4 J/cm^2 and a n_p of 2 were employed. It is possible that a blood vessel can still be coagulated at such a low f_r when n_p is much higher. However, the possibility of collateral skin damage also increases with n_p [26] and thus we did not pursue that line of investigation.

Although the above threshold n_p and f_r might not be optimal for a given PWS treatment, they may represent a

TABLE 2. Summary of the Effect of Pulse Repetition Rate (f_r)

f_r	Number of venules		
	Irradiated	Coagulated	Coagulation %
0.5	4	0	0
1	4	1	25
5	6	5	83

RE = 4 J/cm^2 ; $n_p = 2$.

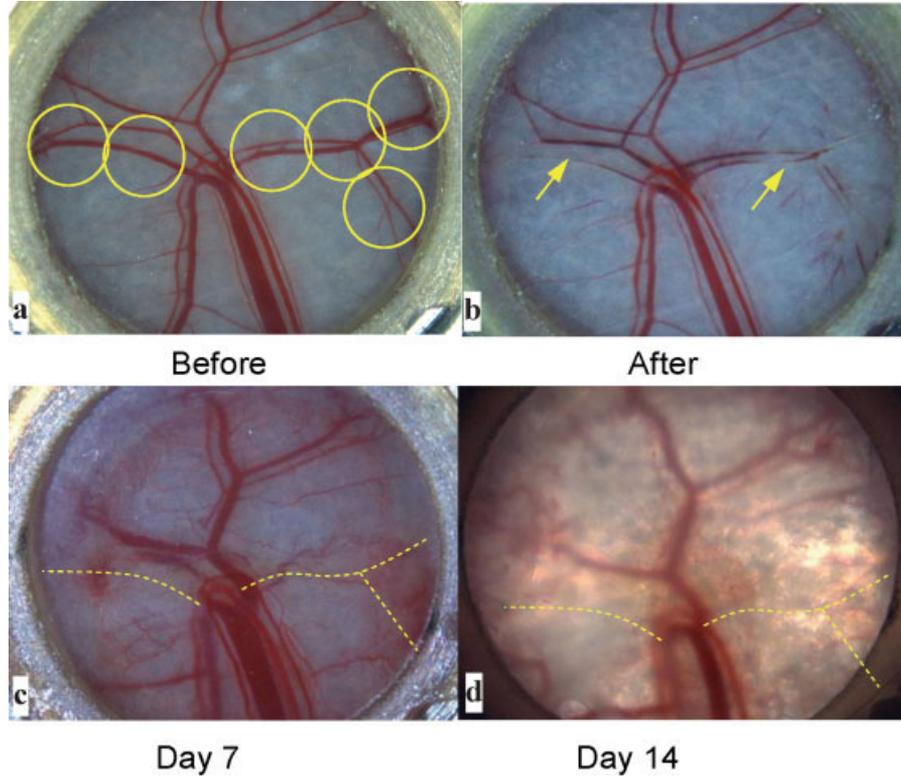


Fig. 4. Long-term response of blood vessels when entire vessel branches were photocoagulated. **a–d**: Color images of the subdermal side of the window before, after laser irradiation, 7 and 14 days later; arrows: photocoagulated blood vessel branches after irradiation; dashed lines: original location of the photocoagulated vessel branches. The photocoagulated branches disappeared completely at Day 14 (d) and thus LSI flow map was not included.

reasonable starting point. While most PDL have a f_r of 1–2 Hz, they could be modified to operate at 5 Hz or higher because lower RE per pulse are required to induce photocoagulation. Another choice is the frequency-doubled Nd:YAG laser used in this study which can produce laser pulses with much higher f_r . However, the epidermal melanin absorption at this laser’s wavelength of

532 nm is higher than that at the customary 585 and 595 nm wavelengths emitted by clinical PDLs.

Due to the limited availability of blood vessels that can be identified clearly in the DWC model, we were only able to determine threshold RE, n_p , and f_r for a range of blood vessel diameters. It is certainly desirable to develop a more detailed correlation between the optimal RE, n_p , f_r , and blood vessel diameter. However, the number of animals that would be required for this purpose could easily become prohibitive. A more practical approach to this problem is to validate current numerical models of laser–tissue interaction using the animal study results and predict the responses of various PWS blood vessels to laser irradiation using such validated numerical models.

Our data on the effect of irradiated vessel length demonstrate that the spatial extent of photocoagulation has considerable influence on the long-term removal of coagulated blood vessels. When a short segment of a blood vessel was photocoagulated, reperfusion of the blood vessel was consistently observed; when the length of the coagulated segment was increased, the probability of long-term removal increased substantially. Our data imply that, besides photocoagulation of the malformed capillary loops in PWS skin, damage of the blood vessels to and from the

TABLE 3. Summary of the Effect of Irradiated Vessel Length

Animal #	Number of branches		
	Irradiated	Reperfused	Reperfusion %
1	1	1	100
2	2	0	0
3	2	0	0
4	2	2	100
5	2	1	50
6	2	1	50
Total	11	5	45

RE = 4 J/cm²; n_p = 5; f_r = 26 Hz.

superficial horizontal plexus using a laser generating a deeper penetrating wavelength will increase long-term PWS therapeutic outcome.

CONCLUSIONS

A MLP approach can induce blood vessel photocoagulation at much lower RE per pulse as compared to SLP. The required f_r of 5 Hz and n_p of 2 are moderately low in terms of modern laser technology which may make the MLP approach practical in the clinical management of PWS. Our results also imply that spatial extent of photocoagulation might have considerable influence on the long-term PWS therapeutic outcome.

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