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Long-Term Blood Vessel Removal With Combined Laser and Topical Rapamycin Antiangiogenic Therapy: Implications for Effective Port Wine Stain Treatment

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Background and Objectives: Complete blanching of port wine stain (PWS) birthmarks after laser therapy is rarely achieved for most patients. We postulate that the low therapeutic efficacy or treatment failure is caused by regeneration and revascularization of photocoagulated blood vessels due to angiogenesis associated with the skin's normal wound healing response. Rapamycin (RPM), an antiangiogenic agent, has been demonstrated to inhibit growth of pathological blood vessels. Our objectives were to (1) investigate whether topical RPM can inhibit reperfusion of photocoagulated blood vessels in an animal model and (2) determine the effective RPM concentration required to achieve this objective.

Study Design/Materials and Methods: For both laser-only and combined laser and RPM treated animals, blood vessels in the dorsal window chambers implanted on golden Syrian hamsters were photocoagulated with laser pulses. Structural and flow dynamics of blood vessels were documented with color digital photography and laser speckle imaging to evaluate photocoagulation and reperfusion. For the combined treatment group, topical RPM was applied to the epidermal side of the window daily for 14 days after laser exposure.

Results: In the laser-only group, 23 out of 24 photocoagulated blood vessels reperfused within 5–14 days. In the combined treatment group with different RPM formulae and concentrations, the overall reperfusion rate of 36% was much lower as compared to the laser-only group. We also found that the reperfusion rate was not linearly proportional to the RPM concentration.

Conclusions: With topical RPM application, the frequency of vessel reperfusion was considerably reduced, which implies that combined light and topical antiangiogenic therapy might be a promising approach to improve the treatment efficacy of PWS birthmarks. Lasers Surg. Med. 42:105–112, 2010. © 2010 Wiley-Liss, Inc.

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

INTRODUCTION

Port wine stain (PWS) birthmarks are congenital cutaneous vascular malformations that occur in an estimated three children per 1,000 live births [1,2]. PWS are light pink and flat in young patients but become red-purple and elevated by middle age. The pulsed dye laser (PDL) [3,4] with epidermal cooling [5,6] is the treatment of choice for PWS, with low incidence of side-effects. However, in the majority of cases complete PWS clearance cannot be achieved even after multiple PDL treatments. Moreover, a significant proportion of lesions remain resistant to laser treatment even at the highest light dosages [7,8]. Novel promising approaches to PWS treatment are needed to achieve better lesion clearance with fewer treatments.

PDL irradiation of PWS skin induces intense blood vessel damage and hypoxia in the dermis as evidenced by intense purpura and histological documentation of vascular wall necrosis [3]. However, the wound healing response often results in regeneration of PWS blood vessels within 1 month

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after laser exposure [9]. Wound healing is a complex, dynamic process in which angiogenesis plays an integral role, since the formation of new blood vessels is necessary to deliver a variety of mediators and regulators [10]. Recent research on mechanisms regulating angiogenesis has identified and exploited several potential agents that can inhibit angiogenesis in pathological conditions such as tumor growth or proliferative diabetic retinopathy [11].

Rapamycin (RPM), an inhibitor of "mammalian target of rapamycin" (mTOR), is a natural macrolide antibiotic derived from *Streptomyces hygroscopicus*. RPM was approved by the Food and Drug Administration (FDA) for use as an immunosuppressant to prevent allograft rejection in organ transplantation [12] and for coating coronary stents to prevent restenosis [13]. Recently, RPM has shown potential in cancer therapy as an antiangiogenic agent, and hence may benefit PWS laser therapy. The antiangiogenic properties of RPM are associated with a decrease in vascular endothelial growth factor (VEGF) production and a reduction in the response of vascular endothelial cells to stimulation by VEGF [14,15]. In addition, RPM inhibits upstream Akt-induced signaling in endothelial cells which reduces vascular permeability and blocks tumor vessel-like vascular morphology [16]. In vitro studies in human smooth muscle and rodent endothelial cells indicate that mTOR inhibition by RPM specifically abrogates hypoxia triggered proliferation and angiogenesis [17]. An in vivo study in mice showed that RPM significantly reduced the extent of neovascularization in both laser induced choroidal neovascularization and hypoxia induced retinopathy of prematurity [18]. Based on these findings, we investigated whether topical application of RPM after laser exposure could inhibit reperfusion of photocoagulated blood vessels in an animal model and determined the effect of RPM concentration on inhibiting reperfusion of photocoagulated blood vessels.

MATERIALS AND METHODS

Animals

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 with an initial bodyweight of 90–120 g were assigned to four groups: laser only ($n = 10$); laser+RPM ($n = 20$); laser+vehicle ($n = 5$), and RPM only ($n = 2$).

Dorsal Window Chamber Model

A dorsal window chamber (DWC) was installed on each animal. This model, first described by Algire in 1943 [19], 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 for 2 weeks (in some cases, chambers have produced clear images for as long as 3–4 weeks) [20,21]. Details of the chamber structure and surgical procedure can be found elsewhere [22,23]. Briefly, after the animal was anesthetized, the dorsal skin was shaved, epilated, and lifted to form a skinfold. A pair of titanium window frames were attached to the front and backsides 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 in diameter and 0.2 mm in 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

In this study, laser irradiation was performed on the window (subdermal) side of the preparation. Blood vessels were irradiated with a frequency-doubled Nd:YAG laser (Dualis^{VP+}, Fotona Laser, Ljublijana, Slovenia) which emits a sequence of a variable number of pulses. The duration of an individual pulse is 1 millisecond and the radiant exposure varies from 3 to 15 J/cm^2 with the 2 mm spot used in this study. The number of pulses could be varied from 1 to 10 and the pulse repetition rate could be varied from 0.5 to 30 Hz. Laser pulse energies were verified using an energy meter (FL250A-SH with Nova display, Ophir, Logan, UT). Both single and multiple laser pulses were used to irradiate blood vessels.

Color and Laser Speckle Imaging

Digital color photos and laser speckle images of the windows were acquired prior to, shortly after laser irradiation and daily thereafter for two weeks. After the day of window implantation, hamsters were anesthetized with a mixture of oxygen and isoflurane (3%) through a nosecone. Reflectance images of the epidermal and dermal sides of the windows were taken with white light illumination. Transilluminated images were taken from the dermal side with green light illumination to achieve better blood vessel contrast. 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, laser speckle imaging (LSI) was used to determine blood flow dynamics in the window [21,24–26]. During LSI, the window was transilluminated with a CW HeNe laser (30 mW) 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 milliseconds with a CCD camera and processed with a sliding-window-based algorithm [27–29] to visualize blood flow in the window.

Topical RPM

Two topical RPM formulae were tested in this study. The first formula contained either 1% or 2% (w/w) RPM powder (LC Laboratories, Woburn, MA) dissolved in 5% benzyl alcohol and thoroughly mixed with a water-based cream (J0757, Conrex Pharmaceuticals, Newtown Square, PA).

The second formula contained 0.5% or 1% or 2% (w/w) RPM powder from the same source dissolved in 5% benzyl alcohol and thoroughly mixed with an ointment (J0969, Conrex Pharmaceuticals). All mixtures were stored at 4°C until use. In both formulae, a skin penetration enhancer (PET™, Conrex Pharmaceuticals) was added. Solvent, skin penetration enhancer and cream/ointment mixture were used as vehicle controls.

Immediately after post-irradiation imaging on Day 0, topical RPM was applied daily to the epidermal side of the window, for 14 days. Each day, the epidermis was cleaned gently with sterile swabs and water to remove any residual RPM. The same procedure was followed for the application of vehicle controls. A plastic cover was loosely attached to the backside of the chamber to prevent unintended loss of the medications.

Data Analysis

Color images and LSI flow maps recorded before and 1 day after laser irradiation were first analyzed to locate photocoagulated blood vessels in which complete flow stoppage occurred. The blood vessels were followed in the color images and flow maps to determine if reperfusion occurred. Epidermal images were also analyzed to determine if adverse cutaneous effects (e.g., skin irritation, scabbing, or ulceration) occurred.

RESULTS

Control Groups

Periodically, a window surgery was performed without further intervention in order to monitor the stability of this animal model. One such window preparation is shown in Figure 1. Although development of granulation tissue hindered the field of view in the reflectance images (top row), there was no significant change in vessel structure and flow dynamics as shown in the transilluminated images (middle row) and LSI flow maps (bottom row). Nevertheless, it can be noted that the window skin did move downward slightly due to the effects of gravity over the course of 14 days.

Two hamsters with DWC were treated with topical RPM (one for each formula) without laser irradiation. In both windows, no major vasculature structural changes were observed on Day 14.

Laser Only Group

There were a total of 10 hamsters in this study group. In each window, a 2 mm segment of all major branches which normally had a pair of arteriole (black arrow) and venule (white arrow) was irradiated with either a single pulse (5–7 J/cm^2) or a series of 5 pulses (3–5 J/cm^2, 26 Hz) (an example is shown in Fig. 2a). The stem of the tree-like vascular network in the window was left intact. Intense photocoagulation of the blood vessels was observed after laser irradiation (Fig. 2b) and blood flow in the irradiated blood vessels was absent in the LSI flow map immediately (Fig. 2f) and 1 day after laser exposure (data not shown). Vasoconstriction and vessel disappearance immediately after laser irradiation were observed [30]. The skin typically became erythematous with the appearance of vascular regeneration and reperfusion around the irradiated sites several days after laser exposure (Fig. 2c,g). On Day 14, the venules appeared similar to those observed before laser irradiation (Fig. 2d,h). However, much less arterioles were observed.

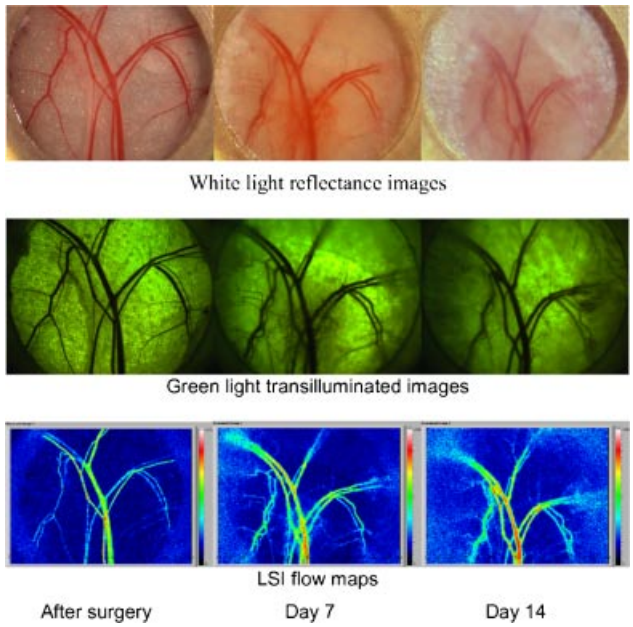

Fig. 1. A window preparation without any intervention. Although development of granulation tissue hindered the field of view in the reflectance images, there was no significant change in vessel structure and flow dynamics as shown in the transilluminated images and LSI flow maps. [Figure can be viewed in color online via www.interscience.wiley.com.]

The results from all 10 hamsters in the laser only group are summarized in Table 1. In total, 30 venules were irradiated and 24 venules were photocoagulated, that is, no blood flow in LSI flow map 1 day after irradiation. Twenty-three out of 24 venules reperfused. Typically, reperfusion occurred between 5 and 14 days after irradiation. The interval between laser exposure and reperfusion did not appear to be related to the radiant exposure applied (data not shown).

The only adverse effect noted in this study group was that skin necrosis on the top corner of the window chambers occurred in several hamsters which might have been caused by insufficient blood supply due to vessel coagulation.

Laser+Vehicle Group

There were a total of seven hamsters in this group. Laser irradiation parameters used for this group were identical to those for the laser-only group. The detailed results from all seven hamsters are summarized in Table 2. In the laser+vehicle group, 16 venules were irradiated and 14 venules were photocoagulated. Thirteen out of 14 venules reperfused. The reperfusion rate was essentially identical to that of the laser-only group.

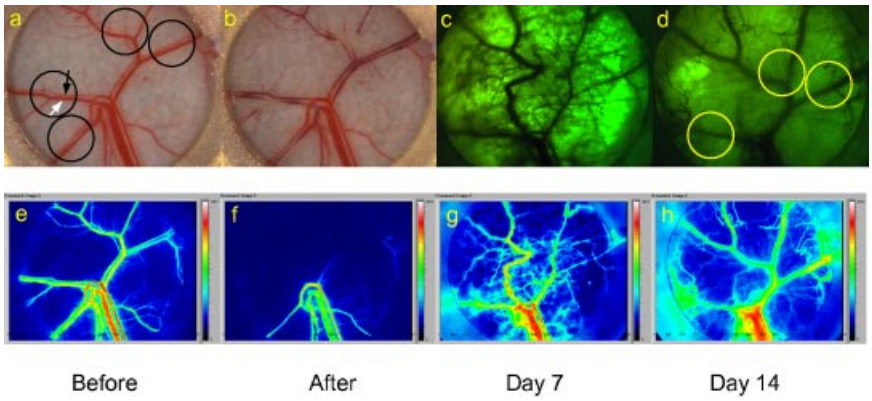

Fig. 2. The reflectance image (a) and LSI flow map (b) of a DWC were shown. Blood vessel photocoagulation was induced by laser pulses (5 pulses, 26 Hz, 3 J/cm^2 per pulse) (**b**,**f**). However, vascular regeneration and reperfusion around the irradiated were seen on Day 7 (c,g) and the irradiated blood vessels reperfused on Day 14 (**d**,**h**). Black arrow: arteriole; white arrow: venule. [Figure can be viewed in color online via www.interscience.wiley.com.]

Laser+RPM Group

There were a total of 20 hamsters in this study group. Laser irradiation parameters used for this group were identical to those for the laser-only group. As shown in Figure 3, photocoagulation of the blood vessels was observed after laser irradiation (Fig. 3b). Blood flow in the irradiated blood vessels was absent in the venules immediately (Fig. 3f) and 1 day after laser exposure (data not shown). At Day 7, the window was erythematous and small blood vessels persisted (Fig. 3c,g). However, those vessels subsequently regressed and reperfusion was not observed in the majority of the coagulated blood vessels on Day 14 (Fig. 3d,h) in this window.

The detailed results from all 20 hamsters are summarized in Table 3. In total, 80 venules were irradiated and 78 venules were photocoagulated. Twenty-eight out of 78 venules reperfused. The overall reperfusion rate of 36% was much lower when compared to the nearly 100% reperfusion rates observed in the laser-only and laser+vehicle groups. The individual reperfusion rate for 16 out of 20 hamsters was within 0–50% and the average reperfusion rate for those 16 animals was only 25%.

TABLE 1. Detailed Results in the Laser-Only Study Group

	Number of venules			Reperfusion
Animal	Irradiated	Coagulated	Reperfused	%
1	2	2	1	50
2	3	3	3	100
3	3	2	2	100
4	2	1	1	100
5	3	1	1	100
6	4	4	4	100
7	2	2	2	100
8	2	1	1	100
9	5	4	4	100
10	4	4	4	100
Total	30	24	23	95

The reperfusion rates for the laser only, laser+vehicle and combined treatment groups with different topical RPM formulae and concentrations are shown in Figure 4. The reperfusion rate for the first formula is higher as compared to second formula with the same concentration. Within the same type of formula, the reperfusion rate is not linearly proportional to the RPM concentration. For the second formula, the reperfusion rate for the 1% RPM ointment was much lower than that for either 2% or 0.5% RPM ointment. The explanation for this phenomenon is not fully understood, however, an in vitro experiment using an aortic ring–vessel sprouting assay also showed a similar trend [31].

Skin necrosis on the top corner of the window chambers was also noted in several hamsters. Some epidermal side irritation around the irradiation sites was observed on most hamsters treated with the first RPM formula. The irritation occurred 2–3 days after laser irradiation and resolved by days 10–12.

DISCUSSION

Our results clearly indicate that persistent reperfusion occurred in photocoagulated blood vessels in this animal model treated by laser only (Fig. 2 and Table 1). However, topical RPM inhibits reperfusion of such vessels in the DWC model (Fig. 3 and Table 3). Reperfusion can be caused by mechanical and biological mechanisms such as flow restoration in incompletely photocoagulated blood vessels, angiogenesis and neovasculogenesis [26,32,33]. Because vessel coagulation was essentially complete in this study, it is hypothesized that angiogenesis/neovasculogenesis is responsible for vessel reperfusion. Shutdown of major branches in the window induced a severely hypoxic microenvironment which can cause overexpression of hypoxia-

TABLE 2. Detailed Results in the Laser + Vehicle Study Group

Laser + vehicle		Number of venules			
Animal	Formula	Irradiated	Coagulated	Reperfused	Reperfusion %
1	1	2	1	1	100
2	1	3	3	2	68
3	1	4	4	4	100
4	2	3	3	3	100
5	2	4	3	3	100
Total		16	14	13	93

inducible factor-1 alpha (HIF-1α) which in turn promoted the secretion of angiogenesis-stimulating factors [34] such as platelet-derived growth factor [35] and VEGF [36]. Therefore, inhibition of the mTOR–HIF-1α–VEGF pathway by RPM is expected to play a major role in preventing vascular reperfusion after laser irradiation. Moreover, the antiangiogenic effect of RPM is also due to a direct antiproliferative response on VEGF-stimulated endothelial cells through inhibition of the PI3K–p70S6 kinase pathway [14,37]. The exact underlying mechanism deserves further study to optimize a combined laser and RPM approach to improve PWS therapeutic outcome.

The nearly 100% reperfusion rate (Table 1) in the laser only group suggests a lower therapeutic efficacy than that is achieved by laser treatment of PWS. The discrepancy might be related to the length of the irradiated blood vessel segment (2 mm in this study as compared to 7–10 mm for PDL). Although further study is needed to determine if a lower reperfusion rate can be achieved when the segment irradiated is longer, it is reasonable to assume that reperfusion would occur and topical RPM would still be required.

The DWC model is one of the few in vivo animal models which permits serial imaging and application of topical agents. Although hamster skin is thinner and contains some elements (e.g., subdermal muscle) not seen in human skin, the ultrastructure of the post-capillary venules within rodent skin is comparable to those in humans because the alteration of PWS vessels is believed to be caused by the progressive ectasia due to a congenital absence of perivascular nerve tissue [38]. The major difference between the hamster skin and non-scalp PWS human skin is that there are numerous hair follicles in hamster skin. Fur regrowth after depilation may alter the window's microenvironment because VEGF expression in hair dermal papilla cells (specialized mesenchymal cells in the hair follicle) was previously reported [39,40]. We noted that fur in the window grew much slower as compared to other shaved and depilated skin when topical RPM was applied. Also of concern with the DWC model is that the direct contact between subdermis and glass window can induce an inflammatory response which may affect the window's microenvironment. This concern can be alleviated by coating the glass window with a thin layer of biocompatible material.

The advantage of topical application is that RPM could be delivered to the dermis while avoiding significant systemic drug absorption and associated side effects [41]. Two different topical RPM formulae were tested in this study. In both formulae, a skin penetration enhancer (PET^{TM}) was used. The results indicate that RPM can be effectively

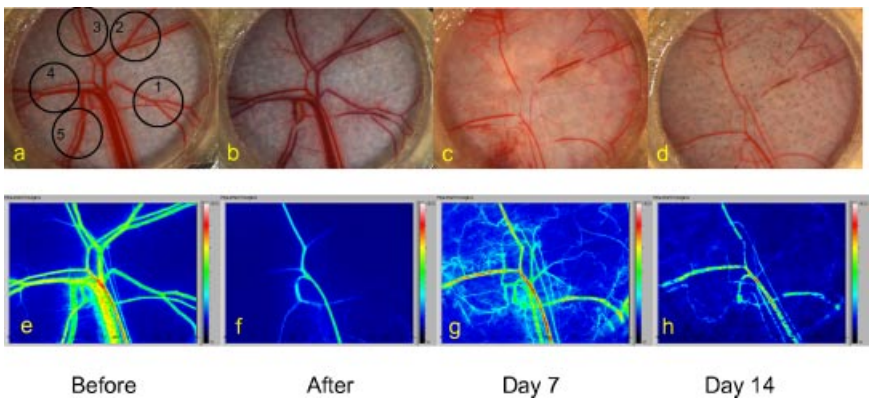

Fig. 3. A DWC treated with combined laser irradiation and 1% topical RPM cream (first formula). Reflectance image and LSI flow map before laser irradiation (**a**,**e**). Intense photocoagulation was induced by laser pulses (5 pulses, 26 Hz, spot 1–3:3 J/cm^2 per pulse; spot 4–5: 4 J/cm^2 per pulse) (**b**,**f**). The window was erythematous and small blood vessels were seen on Day 7 (**c**,**g**). Reperfusion of most coagulated blood vessel was not observed on Day 14 (**d**,**h**). [Figure can be viewed in color online via www.interscience.wiley.com.]

TABLE 3. Detailed Results in the Laser + RPM Study Group

		Number of venules			
Animal	RPM	Irradiated	Coagulated	Reperfused	Reperfusion %
1	[a]F1, 1%	4	4	4	100
2	F1, 1%	6	6	3	50
3	F1, 1%	5	5	0	0
Subtotal		15	15	7	50
1	F1, 2%	2	2	1	50
2	F1, 2%	4	4	1	25
3	F1, 2%	3	3	3	100
4	F1, 2%	4	4	2	50
5	F1, 2%	4	4	0	0
6	F1, 2%	3	3	1	33
7	F1, 2%	4	4	2	50
8	F1, 2%	3	3	1	33
Subtotal		27	27	11	43
1	[b]F2, 0.5%	4	3	1	33
2	F2, 0.5%	4	3	2	67
Subtotal		8	6	3	50
1	F2, 1%	5	5	1	20
2	F2, 1%	4	4	0	0
3	F2, 1%	3	3	0	0
Subtotal		12	12	1	7
1	F2, 2%	5	5	1	20
2	F2, 2%	5	5	2	40
3	F2, 2%	4	4	3	75
4	F2, 2%	4	4	0	0
Subtotal		18	18	6	34
Total		80	78	28	36

[a]F1: first RPM formula.
[b]F2: second RPM formula.

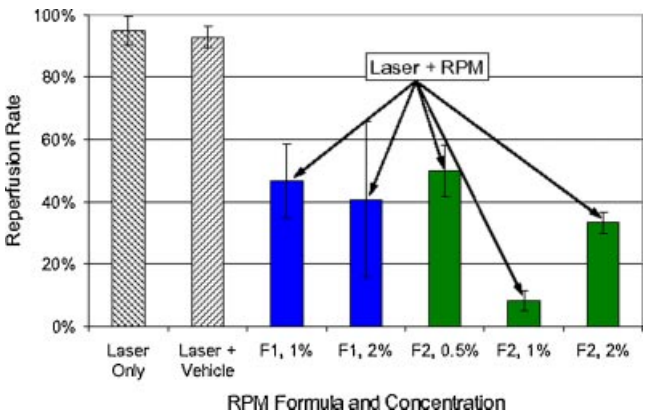

Fig. 4. Reperfusion rates of the coagulated blood vessels on Day 14 for laser only, laser+vehicle and combined treatment groups. F1: first formula; F2: second formula; error bar: median absolute deviation.

delivered through the stratum corneum using this enhancer. In the development of the first formula, emphasis was give to penetration enhancement. Thus, ingredients such as castor oil were used and irritation of laser-irradiated skin resulted. Much milder ingredients were carefully selected for the second formula, and thus, no skin irritation was observed while the delivery of RPM was maintained. Therefore, the second RPM formula appears promising for use in clinical trials on PWS patients.

The period of 14 days to apply RPM onto the DWC was determined by the fact that laser-only blood vessels fully reperfused within such a time frame. How long after laser irradiation should RPM be applied to PWS skin to obtain an optimal antiangiogenic effect? Future work should focus on assessing the flow dynamics of the PWS vasculature following laser irradiation as well as after the combination therapy. Shortly after laser irradiation, perfusion in PWS skin is reduced significantly [42], which may cause local hypoxia and stimulate secretion of angiogenesis-stimulating factors. Perfusion in PWS skin could be

monitored with LSI for an extended period of time. The suggested point to stop RPM application might be the moment when perfusion reaches its plateau.

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