FROM BENCH TO BEDSIDE

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From the American Venous Forum

Histopathologic differences in the endovenous laser ablation between jacketed and radial fibers, in an ex vivo dominant extrafascial tributary of the great saphenous vein in an in vitro model, using histology and immunohistochemistry

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ABSTRACT

Objective: The study aimed to investigate the biologic effects of the 1470-nm endovenous laser (EVL), with a jacketed fiber and a radial fiber, during EVL ablation of an ex vivo dominant extrafascial tributary of the great saphenous vein in our in vitro model by histology and immunohistochemistry.

Methods: Ten segments of the dominant extrafascial tributary of the great saphenous vein were harvested by a consultant vascular surgeon from patients during routine varicose vein surgery. Six segments were treated using an ex vivo model of our design by a 1470-nm EVL with a jacketed fiber. The other four segments were also treated by a 1470-nm EVL but with a radial-firing fiber. Each segment was split into five sections and treated at five different linear endovenous energy densities (LEEDs) at 10 W: 0, 20, 40, 60, and 80 J/cm. The veins were incubated and subsections collected at 6 and 24 hours after treatment. Subsections were immersed in buffered formalin and taken for histologic and immunohistochemical analysis. Histopathologic analysis was then performed.

Results: Treatment with the radial fiber led to a pattern of damage that was more homogeneous than with the jacketed fiber, with no carbonization of tissue present. Significant transmural damage and necrosis were observed at LEEDs of 60 and 80 J/cm in both treatment groups. At the same LEEDs, p53 and caspase 3 analysis showed that transmural cell wall vein death (necrosis or apoptosis) occurred by 6 hours after treatment with both fibers.

Conclusions: There was a significant difference in the effects of treatment with a jacketed fiber and a radial fiber in EVL ablation in vitro. Although both fibers caused transmural vein wall cell death at similar LEEDs, the pattern of damage with the radial fiber was more homogeneous. There was no overtreatment of tissue in terms of carbonization after treatment with the radial fiber. Treatment with the jacketed fiber showed carbonization of tissue at the same LEEDs. (J Vasc Surg: Venous and Lym Dis 2019;7:234-46.)

Clinical Relevance: Endovenous laser ablation has become one of the first-line choices for endovenous thermal ablation of incompetent truncal veins in modern varicose vein surgery. The 1470-nm laser targeted at water as the chromophore has become the "industry standard" for endovenous laser ablation. Currently, the two most commonly used devices are the jacketed fiber and the radial fiber. The jacketed fiber transmits the laser energy as a divergent cone in a forward-firing manner from the tip of the laser fiber, whereas the radial fiber directs the laser beam circumferentially from the tip, hitting the wall at a perpendicular angle. Although clinical studies have been performed previously to see whether one of these devices has an advantage over the other, this study takes away many of the confounding variables and looks at the direct interaction between each laser device and a human vein in an in vitro model. The study has shown that the radial

fiber, when used at the same power and pullback speed, gives a more homogeneous ablation, completely destroying the vein wall at linear endovenous energy densities of 60 J/cm (and maybe less in thin-walled veins) or more, whereas the jacketed fiber gives an asymmetric ablation, with carbonization of part of the wall and full ablation at 60 J/cm or more. By measuring the ablation of the vein wall in terms of necrosis of the cells in the vein wall and apoptosis (programmed cell death), we are able to see the true biologic effect of each device. This in vitro study suggests that the radial-firing laser device may be more effective than the jacketed fiber at lower powers and may give rise to less inflammation because of the lack of carbonization during treatment.

Endovenous thermal ablation (ETA) was first introduced for the treatment of varicose veins in the late 1990s with the advent of bipolar radiofrequency ablation.[1] ETA has been associated with fewer procedural complications, quicker postoperative recovery, and lower varicose vein recurrence than open surgical techniques.[2-9] In 2013, the National Institute for Health and Care Excellence recommended ETA as the first-choice treatment procedure for truncal vein reflux.[10] This lies in common with the guidelines recommended in both the United States[11] and the rest of Europe.[12] Endovenous laser ablation (EVLA), first described by Boné in 1999,[13] is now one of the two most popular methods of ETA for the treatment of incompetent truncal veins.

The aim of EVLA, as with all forms of ablation, both chemical and thermal, is to permanently close the vein. This is achieved by causing a fibrotic closure rather than a thrombotic closure, which might recanalize.[14-16] As sclerotherapy has been performed for decades, many workers have concentrated on the effect of sclerosant on the endothelial cells,[17] imagining the vein walls being "stuck together." However, we have shown that the action of sclerosant on the endothelium actually induces cellular inflammation and apoptosis in the media,[18] and the ablation of small veins with sclerotherapy is likely to be due to this effect on the wall rather than the effect on the endothelium alone.

In truncal veins, with thicker walls, thermal ablation is required to ablate the vein effectively, as can been seen by the poor technical results of truncal vein foam sclerotherapy compared with ETA.[19] For EVLA to be an effective treatment of truncal venous reflux, sufficient energy is required to cause fibrotic occlusion rather than just vein closure by thrombus.[1,20] It was previously believed that the mechanism of action for ETA was induced collagen contraction, followed by fibrosis.[21,22] However, our research group hypothesized that only if transmural vein wall cell death occurs will the target vein subsequently undergo fibrotic occlusion and reflux elimination.[14] We have shown—investigating the thermal spread in tissue in vitro,[23] then confirming the effect on vein ex vivo,[24] and finally using this information clinically—that this approach can result in 100% ablation of treated truncal veins.[25] Hence, we are not interested in the endothelial damage alone but the damage profile to the whole vein wall. A preliminary study from our group examining the effects of the 1920-nm endovenous

laser (EVL) on the vein wall has indicated that complete transmural vein wall cell death, after EVLA treatment, is reliant on both the induction of structural damage and necrosis within the vein wall and the upregulation of apoptosis and inflammation in those cells not directly damaged.[26] Hence, it is not enough to use histology to look for observable thermal damage, but immunohistochemistry (IHC) needs to be used to look for cells undergoing apoptosis.

Various types of EVLs used in EVLA differ by laser wavelength and corresponding target chromophore. These have been shown to vary in clinical effectiveness, cost, and practicality.[22,27,28] In recent years, EVLs targeting water as the chromophore, with wavelengths of 1320 nm, 1470 nm, and 1920 nm, have become the industry standard,[29] replacing EVLs that targeted hemoglobin.[30]

Since the inception of EVLA in 1998, four main types of fiber have been used. However, today, the two most commonly used are the jacketed fiber and the radial-firing fiber. The jacketed fiber projects the laser beam forward in a diverging conical pattern into the lumen and wall of the target vein. The radial fiber emits light circumferentially by way of an optical cone within the tip of the device, directing the beam perpendicularly into the vein wall.[31] There has been much research into the efficacy of the different fibers.[29,32-35] However, there has not been much research into the efficacy of the different fibers at causing transmural vein wall cell death using IHC to look for necrosis and apoptosis.

The objective of this study was to use our in vitro model to analyze and to compare the biologic effects of treating the ex vivo dominant extrafascial tributary (DEFT) of the great saphenous vein (GSV), which, when it is dominant and appears to be the same vein as the GSV but is found extrafascially, is often called the extrafascial GSV, with the jacketed and the radial fiber at different ablation energies. In endovenous surgery, the amount of energy used in treating a vein is called the linear endovenous energy density (LEED), and it is measured in joules/centimeter.[36] Clinically, the power in watts (where a watt is a joule/second) multiplied by the number of seconds taken to pull the device back 1 cm (seconds/centimeter) gives the LEED. In this study, we used different LEEDs with a power level of 10 W with a 1470-nm EVL. Analysis of the effects of treatment on the vein wall was performed by histology and IHC stains and subsequent analyses. With both sets of treatments, a

1470-nm EVL was used, and we thus predicted that changing the fiber type would not dramatically affect the LEED required for transmural vein wall cell death. However, studies have shown that treatment is far more homogeneous with use of a radial fiber than a jacketed fiber.[29,35] Thus, we predicted that the main difference between the two fibers would be the pattern of damage within the vein wall.

METHODS

The study received a favorable ethical opinion from the NRES Committee South East Coast-Surrey (13/LO/0058) and the University of Surrey Ethics Committee (EC/2013/23/FHMS/Fast-Track). All human tissue samples were used and stored at the University of Surrey in accordance with Human Tissue Authority guidelines. Patients' consent was collected, and patients' information was stored in accordance with the Data Protection Act (United Kingdom, 1998).

Ten lengths of the DEFT of the GSV, identified and marked preoperatively by duplex ultrasound, were harvested from consenting patients during routine varicose vein surgery performed under tumescence at the authors' institution. The DEFT of the GSV was surrounded by tumescence and then removed as atraumatically as possible thorough several stab incisions with use of a phlebectomy hook. By cutting one end and pulling through each incision in turn, the vein can be removed in one section. A minimum of 10 cm was required for each vein to be included, and most were about 15 cm long. Immediately after collection, the segments of vein were placed in a transfer medium (Dulbecco modified Eagle medium [GlutaMax; Sigma-Aldrich, Poole, United Kingdom], 10% penicillin-streptomycin [ThermoFisher Scientific, Waltham, Mass], 1% amphotericin B [Thermo-Fisher Scientific], and 0.1% gentamicin [Sigma-Aldrich]) at 37°C. Segments were then cleaned in a sterile environment and cultured (GlutaMax, 1% penicillin-streptomycin, 0.1% amphotericin B) at 37°C with 5% carbon dioxide for 16 hours. The vein segments were cultured immediately after harvest for the venous tissue to stabilize in culture after the trauma of harvest from the body. As the extraction time for each vein varied significantly, if treatment were carried out immediately after harvest, each treated vein would have been outside of ideal culture conditions for extremely varied lengths of time, and thus it is likely that this would affect the subsequently measured levels of phosphoprotein p53 (p53) and caspase 3 (C3). After incubation, segments were treated using our in vitro model, developed to analyze the effects of ETA on the cells of the vein wall.

The segments of vein were then treated in our in vitro model. Briefly, the segments were placed in a Petri dish containing prewarmed culture medium with two holes covered in Parafilm (Sigma-Aldrich) on either side through which four surgical sutures (two on each side)

ARTICLE HIGHLIGHTS

- **Type of Research:** Experimental study using harvested varicose great saphenous vein segments
- **Key Findings:** This study found that 1470-nm lasers with radial fibers led to more homogeneous thermal injury and less carbonization compared with jacketed fibers.
- **Take Home Message:** Radial fiber lasers may be more efficient than jacketed lasers to achieve venous wall necrosis.

were passed and used to stretch the segments to mimic the elasticity of veins seen ex vivo. The EVL fiber was inserted into the lumen of the segment at one end, through one of the holes in the Petri dish, and passed along the lumen of the vein to the point of treatment. Tumescent anesthesia (10 mL of 2% lidocaine with norepinephrine, 5 mL of sodium bicarbonate) was administered directly onto the vein while it lay within the culture medium in case the mixture achieved any constriction around the fiber. Treatment then proceeded.

Six segments were treated in this model by a 1470-nm EVL (VenaCure EVLT; AngioDynamics, Albany, NY) with a jacketed laser fiber (NeverTouch; AngioDynamics). The other four segments were treated by a 1470-nm EVL (A.R.C. Laser GmbH, Nürnberg, Germany) with a radial-firing fiber (A.R.C.). All treatments were carried out in a laser-safe environment. All segments were divided into five sections. One section from each treatment was left untreated to act as a control. Once the laser device had been passed through it (so that it had the same instrumentation as the others), the control section was removed and marked. The other four sections were then treated at 10 W with four different pullback speeds: 2, 4, 6, and 8 s/cm. As each segment was treated, it was marked by passing a polypropylene suture through the end of the treated section, attached to which was an identifying tag. Thus, the five sections taken from each segment were treated with five different LEEDs: 0 (control), 20, 40, 60, and 80 J/cm. We aimed to treat sections of 2 cm at each LEED. Calculations of the total mass of culture medium and total energy given showed that even if the vein itself did not absorb any energy and there were no energy losses from the large surface area of the culture medium, the temperature of the culture medium would rise by only 1.2°C. As such, this was regarded as inconsequential.

Immediately after treatment, each treated vein section was removed from the remaining vein to be treated and halved into two subsections. These subsections were taken for further culturing at 37°C with 5% carbon dioxide. One subsection was harvested at 6 hours after treatment and the other at 24 hours. The reason for this was

that although the direct effect of thermal damage on the structure of the vein will be observable almost immediately on hematoxylin and eosin (H&E) and Martius scarlet blue (MSB) staining, programmed cell death due to apoptosis will develop over time and will be seen by IHC as a change between 6 and 24 hours. On harvesting, subsections were immediately immersed in 10% phosphate-buffered formalin, allowed to fix for 24 hours at ambient temperature, and then processed for histologic and IHC analysis. As each treated vein section had already been halved, histologic sections were taken from the midpoint of each of the halves of the treated section.

Histologic analysis involved staining for H&E and MSB, by standard protocols, as described by Bancroft.[37] IHC analysis was also carried out, by the avidin-biotin-peroxidase method,[38] a method previously used by members of our group.[39] Proteins stained for IHC analysis were smooth muscle actin (SMA) to look for intact smooth muscle cells in the media, p53, and cleaved C3 as markers of apoptosis (programmed cell death). Cells that had undergone coagulative necrosis as a result of the thermal damage were diagnosed as having undergone necrosis by the histopathologist; the cells showed disruption of the normal structure on H&E staining, and on IHC, the smooth muscle cells did not stain with SMA. Those cells undergoing apoptosis looked normal histologically but were identified by the presence of staining for p53 or C3. Of course, once the apoptotic process was complete, these cells were also dead and as such stopped showing any expression of p53 or C3 and started showing signs of necrosis as the nucleus and cellular structure started showing signs of degradation.

Briefly, formalin-fixed samples were cut into 4-μm sections and placed onto charged slides. The sections were dewaxed and rehydrated before blocking of endogenous peroxidase activity by incubation in 3% hydrogen peroxide in methanol, using an automated slide stainer (Tissue-Tek DRS 2000; Sakura, Alphen aan den Rijn, The Netherlands). Epitope demasking was then carried out using a heat digestion technique. Slides were immersed in either pH 6 or pH 9, depending on the primary antibody being used, using citrate buffer (Dako REAL Target Retrieval Solution; Dako, Ely, United Kingdom), and incubated at 95°C for 20 minutes. The

sections were washed with filtered water and the slides mounted onto Shandon coverplates and loaded into Sequenza trays (Shandon Scientific, Runcorn, United Kingdom). The slides were washed with either tris-buffered saline (TBS) or TBS with 0.05% Tween 20 (TBST; Sigma-Aldrich). Universal blocking buffer (Dako), diluted 1:66 in TBS or TBST, was used to block the sections for 20 minutes. The primary antibody was added, diluted in either TBS or TBST according to the requirements of each antibody (Table), and the slides were incubated overnight at 4°C.

After two washes, biotinylated link antibody and link block (normal horse serum; Dako) were added for 30 minutes before another two washes, followed by amplification of binding using avidin-biotin-peroxidase conjugate (Dako). Visualization of the binding was acquired with use of NovaRED (Vector Labs, Peterborough, United Kingdom) before further washing to remove unbound conjugate and counterstaining with Mayer hematoxylin. Finally, the sections were dehydrated, cleared in xylene, and mounted in a mixture of distyrene, a plasticizer, and xylene (DPX; Sigma-Aldrich) for analysis.

Image analysis of sections stained for IHC was carried out by light microscopy and digital image analysis (Nikon NIS BR; Nikon, Tokyo, Japan). Tissues were initially examined at 40× magnification; areas of interest were examined at 100×. Three representative images of the vein wall were taken for each section, the whole vein wall selected as the region of interest, and the percentage of the total region of interest containing positive staining was calculated by the associated software (Nikon NIS-Elements; Nikon) after thresholds had been set. This is called the object area fraction and is expressed as a percentage of the total area being observed. The threshold is the hue, saturation, and intensity range that is assigned, within the NIS-Elements software, as positive staining within the stained tissue. It is similar to red, green, and blue values but was found to be more accurate in our stained tissue at distinguishing between the nonstained and positively stained tissue. Statistical analysis of the results by Kruskal-Wallis analysis of variance followed by post-testing using Dunn multiple comparisons test and Mann-Whiney t-tests was carried out with GraphPad Prism 7 (GraphPad Software Inc, La Jolla, Calif).

Table. Immune markers used for antigen retrieval immunohistochemistry (IHC)

Primary antibody	Antibody type	Supplier	Dilution	Link antibody	Buffer
SMA	Mouse vs human 1A4 (monoclonal)	Dako (Ely, United Kingdom), M0851	1:100	Horse vs mouse 1:1000	TBS
Phosphoprotein p53	Mouse vs human DO-7 (monoclonal)	Dako, M7001	1:50	Horse vs mouse 1:1000	TBST
Cleaved C3	Rabbit vs human 5A1E (monoclonal)	Cell Signaling Technology (Leiden, The Netherlands)	1:1000	Horse vs rabbit 1:1000	TBST

C3, Caspase 3; *SMA*, smooth muscle actin; *TBS*, tris-buffered saline; *TBST*, tris-buffered saline with Tween 20.

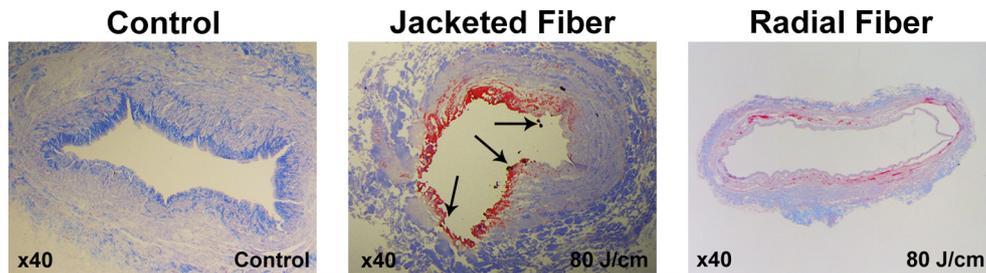**Control**   **Jacketed Fiber**   **Radial Fiber**

Fig 1. Sections of human extrafascial great saphenous vein (GSV) treated by a 1470-nm endovenous laser (EVL) at 80 J/cm (10 W) with either a jacketed or a radial fiber. Sections were stained with Martius scarlet blue (MSB). Collagen is stained *blue* and fibrin *red*. Carbonized tissue appears *black*. Significant asymmetric treatment is seen with the jacketed fiber, with significant carbonization of tissue. No evidence of this is seen in the sections treated with the radial fiber.

RESULTS

All 10 segments of the DEFT of the GSV were successfully treated with EVLA 1470-nm EVLs with the different fibers in our in vitro model. Histopathologic evaluation showed a clear increase in the direct thermal damage to the vein wall of the GSV as the LEED increased. Direct damage refers to the disruption of the vein wall seen on H&E staining and fibrin deposition on MSB staining (Fig 1) as opposed to delayed damage that would be revealed by the presence of apoptotic proteins by IHC (Fig 2). Analysis of the damage profile showed a clear difference in the thermal damage caused by the jacketed and radial fibers. A greater homogeneity of treatment was observed with the radial-firing fiber in the distribution of the damage seen on H&E staining and fibrin on MSB staining (Fig 1). A significant proportion of samples treated with the jacketed fiber showed asymmetric treatment, which was not identified in any vein treated with the radial-firing fiber (Fig 1).

In tissue treated with the jacketed fiber, H&E sections showed significant structural damage and necrosis in sections treated at 60 and 80 J/cm that penetrated throughout the layers of the vein wall. In sections treated at 40 J/cm, there was limited tissue damage and necrosis within the inner media of the vein wall. MSB staining confirmed this and showed significant levels of fibrin
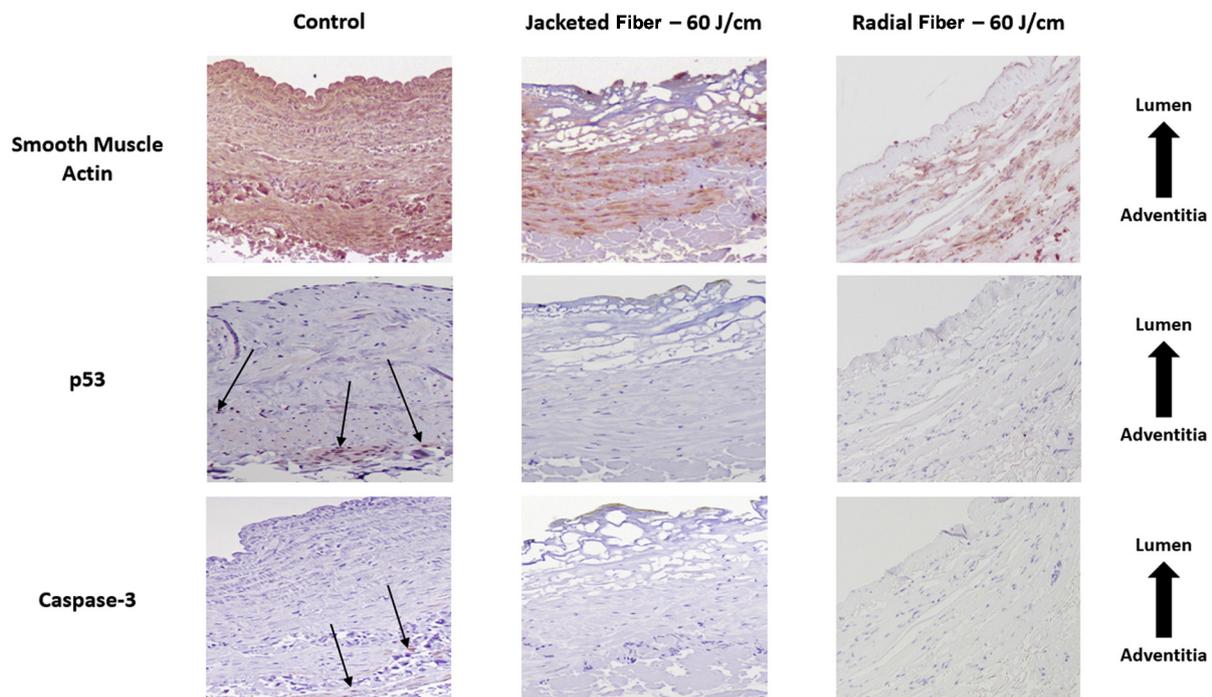

Fig 2. Sections of human extrafascial great saphenous vein (GSV) treated by a 1470-nm endovenous laser (EVL) at 60 J/cm (10 W) with either a jacketed or a radial fiber. Stained immunohistochemically for smooth muscle actin (SMA), p53, and caspase 3 (C3). Sections were harvested at 6 hours after treatment. The *brown-red* color indicates positive expression; the *arrows* indicate examples of expression for p53 and C3 stains (magnification ×100).
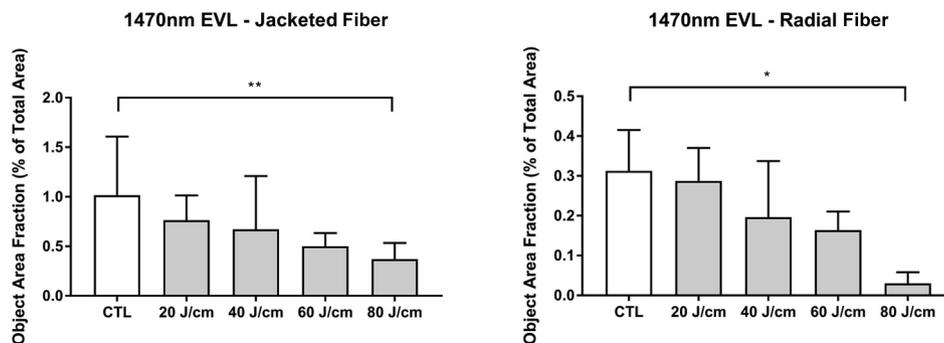
Fig 3. Expression of smooth muscle actin (SMA), shown as the percentage of the total area of the vein wall that stained positive for SMA, in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (*EVL*; 10 W) with either a jacketed or a radial fiber at different linear endovenous energy densities (LEEDs). Statistical analysis by Kruskal-Wallis analysis of variance for the group treated with the jacketed fiber showed a significance of .0088. Post-testing by Dunn multiple comparison testing showed a significance only between the control (*CTL*) and sections treated with 80 J/cm (**P = .0076; n = 12). Statistical analysis by Kruskal-Wallis analysis of variance for the group treated with the jacketed fiber showed a significance of .0317. Post-testing by Dunn multiple comparison testing showed a significance only between the CTL sections and those treated at 80 J/cm (*P = .0317; n = 6).

deposition and carbonized tissue at LEEDs of 60 and 80 J/cm (Fig 1). In some sections, this was visible at 40 J/cm.

In tissue treated with the radial fiber, H&E sections showed a pattern of damage that was similar to the treatment with the jacketed fiber, with transmural necrosis and structural damage visible at treatment LEEDs of 60 and 80 J/cm. However, this damage was more homogeneous around the vein wall. In some veins with thinner vein walls, transmural damage and necrosis were seen in sections treated at 40 J/cm. MSB staining showed significant levels of tissue destruction in the sections treated at 40, 60, and 80 J/cm (Fig 1). The area of damaged vein wall was more significant in the sections treated with at least 60 J/cm. Both the H&E and the MSB stains showed no evidence of any carbonization of tissue at any treatment LEED.

These histologic observations were confirmed with α-SMA IHC staining. Treatment of a vein with a 1470-nm EVL led to the increasing degradation of smooth muscle as the LEED increased, independent of which fiber was used. Accounting for asymmetric treatment, histopathologic evaluation showed that there was little difference in the degradation of SMA in both treatments (Fig 2). Quantification of the SMA expression in the treated tissue was carried out, and statistical analysis showed that treatment with the jacketed fiber had a significant effect on the expression of SMA (*P* = .0088). Treatment with the radial fiber also showed a significant decrease in the expression of SMA as LEED increased (*P* = .0317; Fig 3). For both fibers, post-testing showed significance only between the controls and the sections treated with 80 J/cm in both treatments.

Histopathologic analysis of both α-p53 and α-C3 IHC staining showed that control sections exhibited a baseline expression of p53 and C3 that varied dramatically from vein to vein. This is probably a result of the method of removal of the vein when it was first harvested from the patient. Areas that had been instrumented or pulled are more likely to show increased levels of p53 and C3. In the quantified data, this resulted in large standard deviations within the two control groups, which was carried forward in the treated tissue groups.

Treatment with the radial fiber, at both time points, resulted in a decrease of p53 expression across the vein wall as the treatment LEED increased. At LEEDs above 40 J/cm, there was limited or no expression of p53 in any of the sections. Statistical analysis of the samples harvested at 6 hours showed a significant decrease in p53 expression as the LEED used to treat the sections increased (*P* = .0364). Post-testing showed significant differences between the control and the sections treated at 60 J/cm (*P* = .0116) at the same time point. In comparing each treatment LEED at 6 and 24 hours, no significances were observed (*P* > .05; Figs 2 and 4). A similar pattern of results was observed after treatment with the jacketed fiber; the samples harvested at 24 hours showed a significant decrease in p53 expression as LEED increased (*P* = .0313), and post-testing showed significant differences between the control and the treatments at 60 and 80 J/cm (*P* = .0471 and .096, respectively) at the same time point.

Treatment with the radial fiber resulted in a decrease of C3 expression at all treatment LEEDs. Although some expression was seen in sections treated at 20, 40, and 60 J/cm, little or no expression was seen at a treatment LEED of 80 J/cm. Statistical analysis of the samples harvested at 6 hours showed a significant decrease in C3 expression as the LEED used to treat the sections
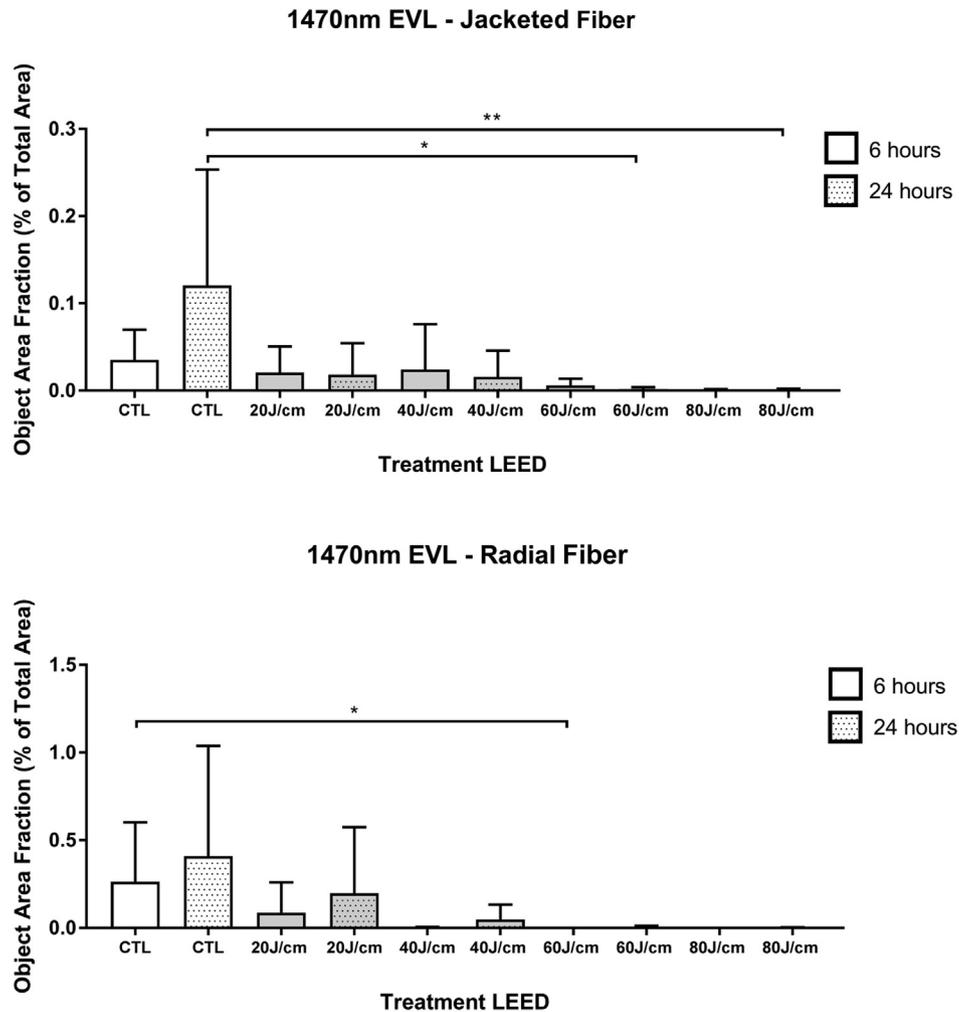
1470nm EVL - Jacketed Fiber

1470nm EVL - Radial Fiber

Fig 4. Expression of p53, shown as the percentage of the total area of the vein wall that stained positive for p53, in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (*EVL*; 10 W) with either a jacketed or a radial fiber at different linear endovenous energy densities (*LEEDs*). Statistical analysis of each time point by Kruskal-Wallis analysis of variance for the group treated with the jacketed fiber showed a significance for the 24-hour time point of .0313. Post-testing by Dunn multiple comparison testing at that time point showed significances between the control (*CTL*) sections and the sections treated at 60 and 80 J/cm (*P = .0471 and **P = .096, respectively). Analysis of the 6-hour time point showed no significances in the Kruskal-Wallis analysis of variance or the Dunn post-testing. Mann-Whitney *t*-tests comparing each treatment LEED at 6 and 24 hours showed no significances (n = 5). Statistical analysis of each time point by Kruskal-Wallis analysis of variance for the group treated with the radial fiber showed a significance at the 6-hour time point of .0364. Kruskal-Wallis analysis of variance for the 24-hour time point group did not show a significance. Post-testing by Dunn multiple comparison testing showed a significance in the 6-hour time point group between the CTL sections and those treed at 60 J/cm (P = .0116) but not for any others. Mann-Whitney *t*-tests comparing each treatment LEED at 6 and 24 hours showed no significances (n = 4).

increased (P = .0148). Post-testing showed significant difference between the control and the sections treated at 40 J/cm (P = .0097) at the same time point (Figs 2 and 5). By comparison, treatment with the jacketed fiber resulted in a decrease of C3 expression at 40, 60, and 80 J/cm but not at 20 J/cm. At 20 J/cm, C3 expression was similar to the controls. At both time points, even though a visual trend was visible, both in the data and in the histopathologic analysis, increasing the LEED did not significantly affect the expression of

C3, at both time points, with use of the jacketed fiber (P > .05). At 6 hours, the markers of apoptosis (p53 and C3) are increased in the vein walls in which apoptosis has been stimulated. However, by 24 hours, apoptosis has been completed in most of these cells, resulting in cell death and hence disappearance of the proteins expressed during apoptosis. This is shown in Figs 6-9, which depict the same data as in Figs 3-5 but normalized to 100% to show the changes more clearly.
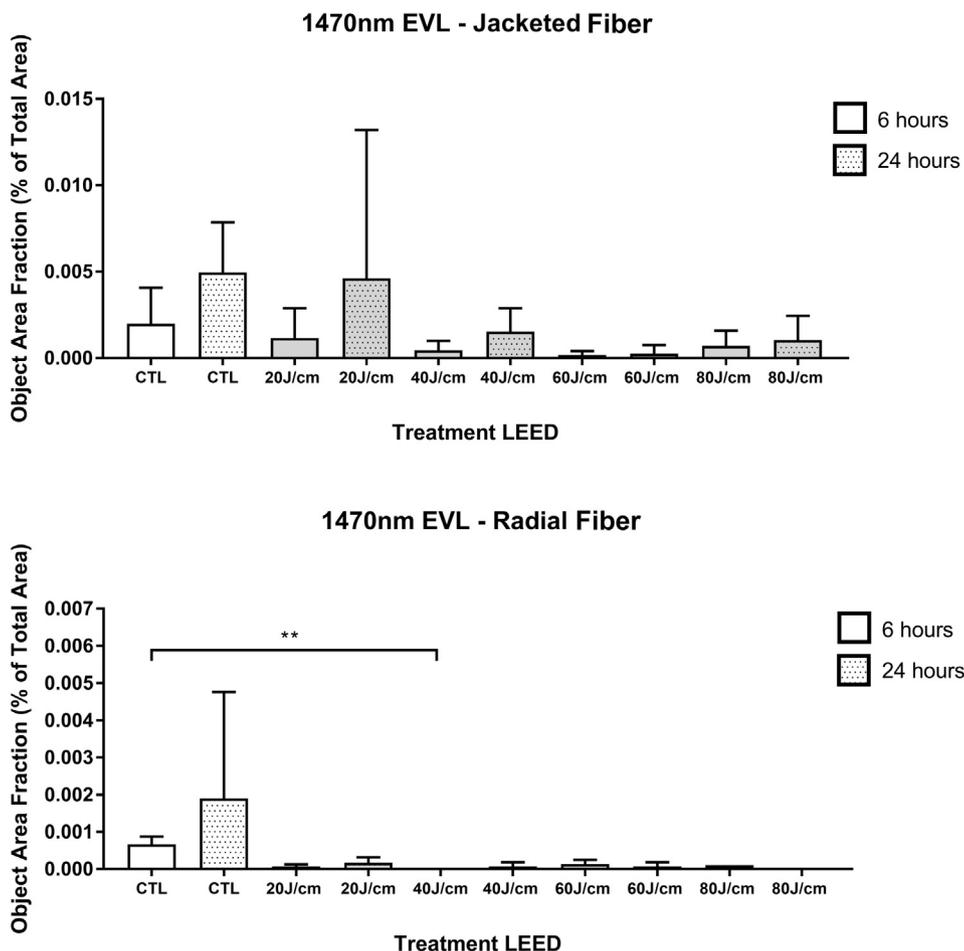
Fig 5. Expression of caspase 3 (C3), shown as the percentage of the total area of the vein wall that stained positive for C3, in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (*EVL*; 10 W) with either a jacketed or a radial fiber at different linear endovenous energy densities (*LEEDs*). Statistical analysis of each time point by Kruskal-Wallis analysis of variance for the group treated with the 1470-nm EVL showed no significance for either. Post-testing by Dunn multiple comparison testing showed no significances. Mann-Whitney *t*-tests comparing each treatment LEED at 6 and 24 hours showed no significances ($P > .05$; n = 4). Statistical analysis of each time point using Kruskal-Wallis analysis of variance for the group treated with the radial fiber showed a significance at the 6-hour time point of .0148. Kruskal-Wallis analysis of variance for the 24-hour time point group did not show a significance. Post-testing by Dunn multiple comparison testing showed a significance in the 6-hour time point group between the control (*CTL*) sections and those treated at 40 J/cm (****P = .0097**) but not for any others. Mann-Whitney *t*-tests comparing each treatment LEED at 6 and 24 hours showed no significances (n = 3).

Thus, in summary:

• Jacketed: At 80 J/cm, transmural vein wall death was seen in most sections except for one section with a thick vein wall, where some viable tissue remained in the half of the vein wall that was not directly treated. At 60 J/cm, the same story applies. At 40 J/cm, there was little or no evidence of complete transmural vein wall death in the sections of the treated veins.

• Radial: At 80 J/cm, in all sections treated with the radial fiber, we observed little or no expression of p53 or C3 at 24 hours after treatment, indicating that transmural vein wall cell death had occurred. At 60 J/cm, in large-diameter veins, there is an indication that viable tissue remains, shown by C3 expression, at 24 hours after

treatment, but smaller diameter veins have undergone transmural death. At 40 J/cm, viable tissue remains in all tissue except the smaller diameter vein walls, where there was evidence of transmural vein wall death at 24 hours after treatment.

DISCUSSION

In comparing the effects of 1470-nm EVL treatment with two different fibers, jacketed and radial, in terms of structural damage to the vein wall, the results indicated a slight difference in terms of the minimum LEED required to achieve substantial transmural damage and necrosis. Results with the radial fiber showed that a LEED as low as 40 J/cm at 10 W is sometimes sufficient to cause
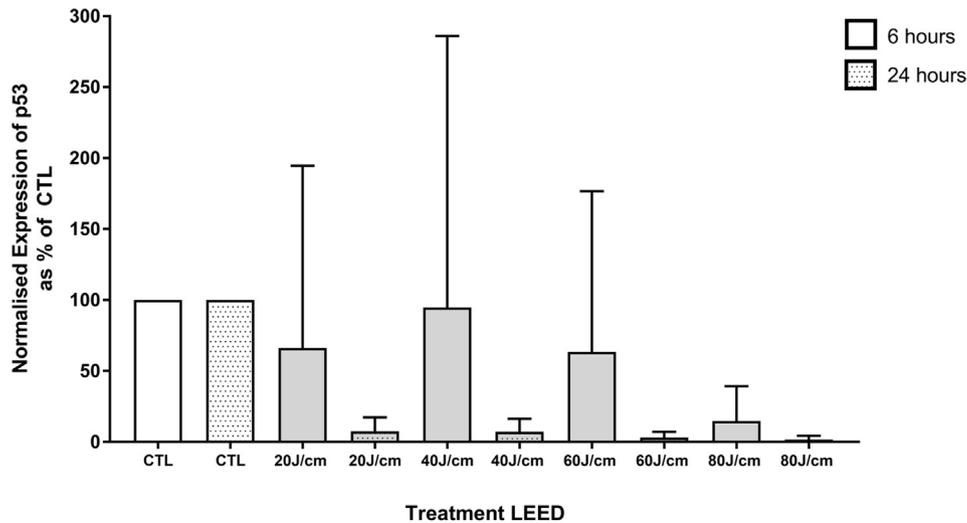
Fig 6. Normalized expression of p53 in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (EVL; 10 W) with a jacketed fiber at different linear endovenous energy densities (*LEEDs*). Expression of p53 at various LEEDs is shown as a percentage of the control (*CTL*) sections (n = 5).

significant transmural penetration of thermal necrosis. No evidence of this is seen after treatment with the jacketed fiber. This indicates that the radial fiber may be more efficient than the jacketed fiber at treating the GSV. Both sets of results indicate that a LEED of 60 J/cm or higher is required to always cause significant transmural penetration of thermal necrosis and structural damage throughout the vein wall in our in vitro model.

The biggest difference between the two fibers is the pattern of damage in the vein wall. The damage in the wall treated with the jacketed fiber is heterogeneous and asymmetric in nature, with the damage being concentrated in half of the vein wall and very little damage observed in the other half. By comparison, treatment with the radial fiber leads to much more homogeneous treatment, with a similar extent of damage visible around the whole of the vein wall (Fig 1). Although this was observed in an in vitro model using ex vivo tissue, it is likely that this would also occur during actual treatment in vivo. Asymmetric treatment is more likely to lead to unsuccessful treatment as a large part of the vein wall remains structurally undamaged and viable tissue remains there. Potential risks associated with EVLA, such as perforation and subsequent ecchymosis, are also indicated to be more likely in treatment with the jacketed fiber as all energy is being applied to one area of the vein wall as opposed to being spread evenly around the vein wall.
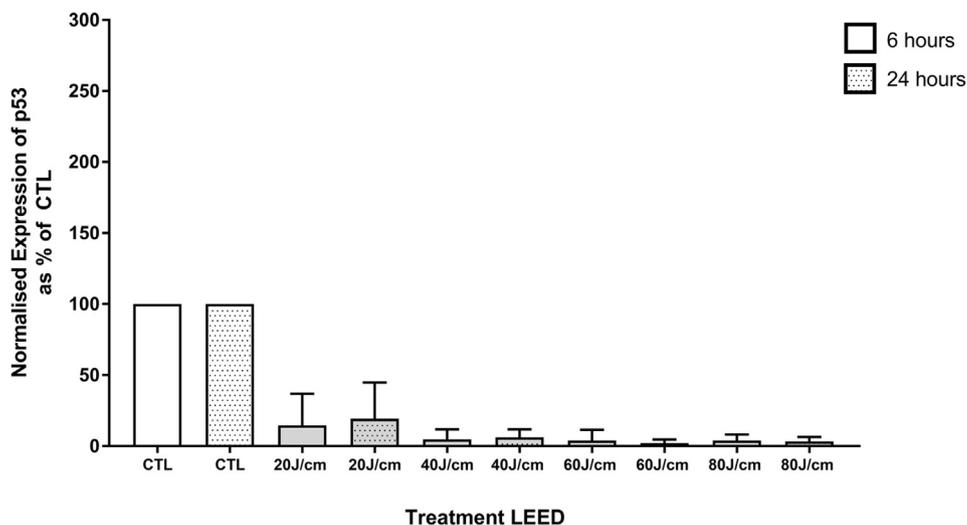

Fig 7. Normalized expression of p53 in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (EVL; 10 W) with a radial fiber at different linear endovenous energy densities (*LEEDs*). Expression of p53 at various LEEDs is shown as a percentage of the control (*CTL*) sections (n = 4).
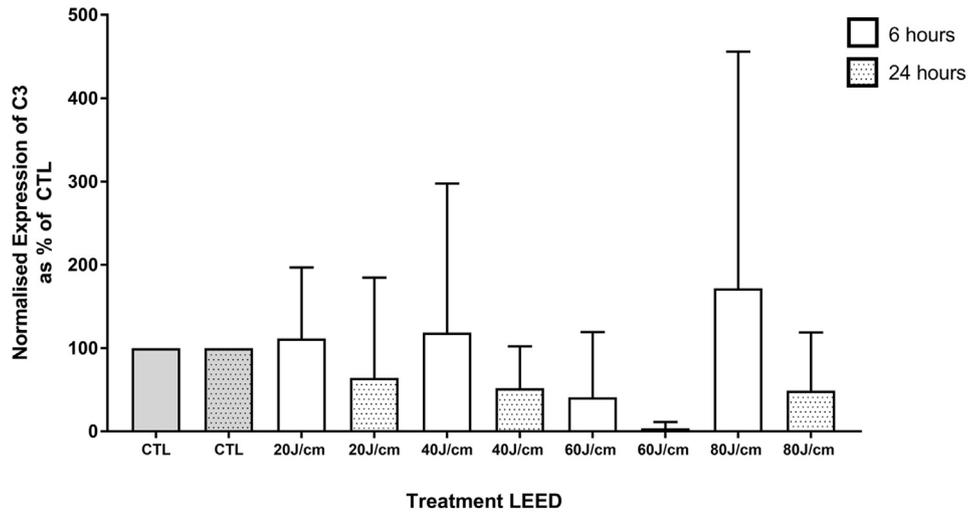
Fig 8. Normalized expression of caspase 3 (*C3*) in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (EVL; 10 W) with a jacketed fiber at different linear endovenous energy densities (*LEEDs*). Expression of C3 at various LEEDs is shown as a percentage of the control (*CTL*) sections (n = 4).

Another significant difference between the two fibers is the presence of carbonized tissue within the vein wall in tissue treated at high LEEDs (60 and 80 J/cm). Significant carbonization was seen after treatment with the jacketed fiber, but no evidence of carbonization was seen in the sections treated with the radial fiber (Fig 1). When tissue is carbonized, it has been overtreated, and thus it can be considered a waste of energy that could have been applied to other areas of the vein wall. The presence of carbonized tissue in vivo may cause a significant inflammatory response, above that of necrosis alone, and therefore could potentially be a cause of increased pain to the patient.

Statistical analysis of the quantified expression of SMA did show a difference between the two groups. In terms of the degradation of SMA as LEED increased, statistical analysis showed a significance of .0088 for the sections treated with the jacketed fiber and .0317 for the radial fiber. This indicates that treatment with the jacketed fiber may be able to damage the smooth muscle of the vein wall more significantly than the radial fiber.

In terms of the pattern of apoptotic expression, there is a slight difference between treatments with the two fibers. In both groups, as the LEED increased, the expression of both p53 and C3 fell at both the 6-hour and the 24-hour time points, with very little expression seen at
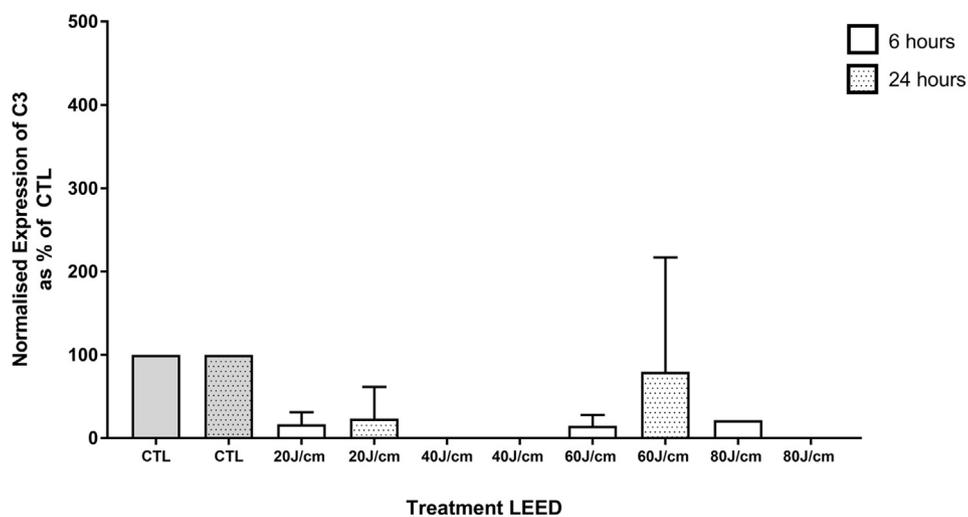

Fig 9. Normalized expression of caspase 3 (*C3*) in extrafascial great saphenous vein (GSV) treated in vitro by a 1470-nm endovenous laser (EVL; 10 W) with a radial fiber at different linear endovenous energy densities (*LEEDs*). Expression of C3 at various LEEDs is shown as a percentage of the control (*CTL*) sections (n = 3).

60 or 80 J/cm. This decrease in apoptotic proteins is due to increasing numbers of cells dying with higher LEEDs. Statistical analysis was similar between the two groups for both the p53 and C3 analysis. Overall, the results indicated that treatment by the 1470-nm EVL, with either a jacketed or radial fiber, at a LEED of at least 60 J/cm causes total vein wall death at 6 hours after treatment. In some cases, with veins with smaller than average vein wall diameters, treatment with the radial fiber at 40 J/cm results in total vein wall death by 6 hours after treatment. Indeed, the lack of p53 at a LEED of 40 J/cm with use of the radial fiber and no expression of C3 at 80 J/cm showed that the homogeneous heating of the vein wall with a radial fiber caused direct necrosis of the cells. If the cells had only been injured, the apoptotic proteins would have been seen.

As with all research projects, there are several limitations of this study that must be taken into account in interpreting these data and attempting to apply them to the clinical situation. Some workers in this field use blood in the vein to simulate "physiologic conditions" of endovenous surgery.[40,41] However, we have recently performed a study looking for how much intraluminal blood there is during EVLA. When the patient is in the Trendelenburg position, with tumescence in place and an EVLA device in situ, there is no measurable intraluminal blood in the vein being treated (unpublished data). Hence, with a 1470-nm laser that uses water as its main chromophore, we believe that we are justified in not adding any blood to the model.

CONCLUSIONS

Overall, the histologic and IHC analysis appears to show that treatment of the ex vivo DEFT of the GSV with the radial fiber shows a more homogeneous destruction of the vein wall compared with the jacketed fiber by a 1470-nm EVL. For successful transmural vein wall death to take place after treatment, all the cells in the vein wall must be either dead or undergoing apoptosis soon after treatment. Assuming complete exsanguination of the vein, the results indicate that both fibers can cause transmural vein wall death at LEEDs above 60 J/cm in vivo. The radial fiber may be able to cause transmural vein wall death, in some cases, at a LEED as low as 40 J/cm. However, the pattern of damage is far more homogeneous after treatment with the radial fiber, and there is no evidence of carbonization of tissue that would, in vivo, result in a higher chance of successful ablation and treatment at this lower LEED. Of course, this is a laboratory experiment rather than a study of veins treated in vivo, and so whether this translates to a clinical significance is for other studies to investigate.

Finally, as stated before, this is an ex vivo experiment in a laboratory model rather than a clinical finding. The use of the ex vivo model allows us to control the environment and treatment as well as to study the tissue without having to perform a subsequent excision of the treated vein from the patient, with resulting scarring and additional procedures. Although human vein was used, there are some obvious limitations to this study. As stated in the Methods section, the vein used was not the GSV but rather the DEFT of the GSV. As with all veins, the wall thickness of the veins used varied, as would be expected between different subjects. We used culture medium rather than blood as explained before as we have previously shown that in the head-down position with tumescence, there is no significant blood in the treated vein. Perhaps the most significant limitation of the laboratory model is that there is no intact immune system. Therefore, we can show the damage to the vein that will stimulate the immune response, but we cannot actually observe the immune response itself.

AUTHOR CONTRIBUTIONS

Conception and design: MW
Analysis and interpretation: HA, ED, FS, JN, RLR, MW
Data collection: HA, FS, MW
Writing the article: HA, ED, MW
Critical revision of the article: FS, JN, RLR, MW
Final approval of the article: HA, ED, FS, JN, RLR, MW
Statistical analysis: HA, FS
Obtained funding: RLR, MW
Overall responsibility: MW

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