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Photodynamic Therapy Versus Glucose for the Treatment of Telangiectasia: A Randomised Controlled Study in a Rabbit Ear Model

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WHAT THIS PAPER ADDS

This experimental animal study shows that photodynamic therapy (PDT) of telangiectasia significantly reduces the treated vein length and area temperature without causing severe histological damage, such as necrosis or ulceration. These preliminary findings should stimulate further tests using this new approach for the treatment of telangiectasia.

Objectives: Telangiectasia is a common venous formation that mainly affects women and causes discomfort, including psychological distress. This study compared photodynamic therapy (PDT) with glucose for vessel sclerosis in a rabbit ear model.

Methods: Thirty-six ears of 18 rabbits were randomly divided into four groups: Group 1: only injection of Photogem (4 mg/mL); Group 2: only light (635 nm, 100 mW/cm^2, 8 min, 48 J/cm^2); Group 3: glucose 75% injection; Group 4: PDT procedure with injection of Photogem and illumination immediately after. Injections were made into the central ear artery. After injection or sham procedures, manual compression of the marginal vein was maintained for 8 min in all ears. Follow up was immediately after the procedures, and one and six days later. The percentage of length reduction of spider veins, the target vessels, was analysed in digital photographs with Image J software. Ear thermographs were made with a thermocamera device and average temperatures were collected for analysis. Ear biopsies were obtained after six days. Endothelium average, inflammation, fibrosis, necrosis, skin burn, and vascular thrombosis were assessed using a specific score.

Results: The mean vessel length reduction was 26% for Group 4, 2.4% for Group 3, .4% for Group 1, and 0 for Group 2, highlighting that in Group 4, the vessel lengths were significantly reduced compared with the other groups ($p < .001$). In the thermal analysis, in Group 3, the temperature was unchanged from the initial temperature and the central diameter vessel increased after six days, while, in Group 4, the temperature decreased and the vessels were not clearly detected, suggesting a reduction of the vessels and smaller infusion. Histology showed no difference among groups and one case of necrosis was found in Group 4.

Conclusions: PDT was associated with significantly more target vessel sclerosis than glucose injection and controls.

Keywords: Hypertonic glucose solution, Photodynamic therapy, Rabbit ear model, Telangiectasia
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INTRODUCTION

Telangiectasia or spider veins are small dilated blood vessels that mainly affect women and cause discomfort, local pain and/or psychological distress. They can be found in many anatomical sites, and are classified as Class 1 according to the system of classification for chronic venous diseases (CEAP).[1]

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Although the conventional treatment consisting of intravenous injection of glucose of 50%/75% (in Brazil) or polidocanol (in developed countries) has good efficacy, several minor complications such as venous ulcers or pigmentation can occur. Transcutaneous laser is a therapeutic alternative, but it can cause skin dyschromias, ranging from hypopigmented spots to focal hyperpigmentation, burns, and ulcers, depending on the intensity of the released energy and skin type. However, there is no technique that guarantees complete elimination of telangiectasia.[2,3]

Photodynamic therapy (PDT) is proposed as an alternative, in which a photosensitiser (PS) is administered locally or systemically and, after biodistribution and vascular accumulation, the local area is then illuminated with an appropriate wavelength of light for PS absorption and subsequent photodynamic reaction. Absorption of light by the PS starts photochemical reactions that generate cytotoxic products (reactive derived oxygen species), which cause cell death.[4,5]

Damage from PDT can occur in various ways, depending on the cellular target reached.[6] Vascular damage can result from blood flow stasis on initiation of light application.[7] Oxygen derived reactive species (ODRS) are important elements of the PDT effect, and, therefore, are fundamental for the efficiency of the technique. The potential vascular damage after PDT is well established in the literature and tissue ischaemia or microvascular occlusion and necrosis after illumination of photosensitised tissue are the most common observations reported.[8−11]

The main goal of the present study was to compare efficacy and safety of PDT with a standard treatment with hypertonic glucose in reducing telangiectasia like vessels in rabbit ears.

METHODS

Experimental groups

The rabbit ear model was chosen because the blood vessel diameter is similar to that of human micro-varicose veins. An open randomised study was designed, and the Institutional Animal Care and Use Committee (protocol 13/2014) approved all procedures. All experiments followed the US National Institutes of Health and European Commission guidelines.

A total of 36 ears of 18 healthy Norfolk rabbits (average weight 2 kg) were randomised using a sequential random number generator in computer software. The numbers were related to the groups and were stored in sequential sealed envelopes. The rabbit ears were divided into the following groups, with nine ears each:

Group 1: Control A, only injection of PS;
Group 2: Control B, only light, using the same parameters of illumination for PDT;
Group 3: Treatment with glucose 75% injection;
Group 4: Treatment by PDT.

All animals were anaesthetised with sodium phenobarbital (Hipnol, 20 mg/kg) intravenously. Before all procedures, the hair on the area of interest of each ear was removed with cream and a razor blade. Injections of glucose or PS solutions were made into the ear central artery at the volume necessary to fill all other ear vessels until whitened (about 3 mL). After injections or during light exposure, 8 min of marginal ear vein manual compression (for local blood flow blockage) was performed in all animals.

Images were taken before, immediately after, one day, and six days after the procedures. The animals were euthanised at the end of the experiment, by injection of KCl, and following ethics committee guidelines.

PDT

Photogem, a haematoporphyrin derivative, was diluted in saline solution (4 mg/mL) and injected into the central ear artery of anaesthetised animals, using a 3 mL syringe and 27 g $\times$ ½ inch needle. After injection and manual compression of the marginal vein in the distal end of the ear, an area of 2 $\times$ 2 cm^2 was illuminated with an arrangement of LEDs of LINCE devices (MMOptics, Brazil), emitting at 635 nm, with fluency of 100 mW/cm^2 ever 8 min, delivering a total superficial dose of 48 J/cm^2.

Image acquisition

High definition (>300 dpi) digital photos were taken with a camera (model Sony DSC-H50) with ear transillumination. Thermal images were obtained using a thermal imager (Fluke FLK-Ti400 model), with thermal sensitivity $\leq$ .05 °C and 320 $\times$ 240 pixel resolution. The images were taken immediately after the procedures, and after one and six days (after again removing hair from the ear).

Thermal image processing

The thermal images were processed using MATLAB software (version R2015a) (MATrixLABoratory, MathWorks, USA), which presented the temperature average of the demarcated region. An area in which the procedure was performed outside the main central artery was defined for each case. In Groups 2 and 4, the illumination area was selected. As the injection groups have no defined area, the same region of ear as the PDT procedure was chosen on the ears of rabbits in Groups 1 and 3.The ambient temperature of the room was controlled by air conditioning at 25 °C, and this was maintained for all animals during image acquisition.

Histology

After the end of the observation period (six days), the animals were euthanised and ear biopsies with a size of about 9 cm^2, related to the PDT are, were obtained and processed for histology for all groups. The same region of the ear was selected for all groups, using the area of PDT treatment. The analysis was performed with haematoxylin eosin (HE) and Masson trichrome dyes and was classified by a pathologist who had no knowledge of the groups. The parameters evaluated were endothelium, inflammation, fibrosis, necrosis, skin burn, and vascular thrombosis through specific scores: inflammation and architectural distortion analysis followed the score given in Table 1; for ulceration, granulation tissue, hyperkeratosis, parakeratosis, thrombosis, epithelial micro-abscess, subepithelial hyalinisation, subcutaneous fibrosis, necrosis, and neovascularisation, 0 was given if absent and 1 if present.

Histochemical analysis

The collagen fibres were analysed by picrosirius red staining and quantified by a morphometric method, as described previously.[12] Briefly, 10 random images from histochemical

Table 1. **Histological assessment of efficacy of telangiectasia treatment: Inflammation and distortion score**

	0	1	2	3	4
Degree of inflammation	Absence	Discreet	Notable	Intense	Abscess
Type of inflammation	Without	Neutrophilic	Lymphocytic	Mixed	—
Architectural distortion	No	Follicular	Nervous vasculature	Subcutaneous	—

staining slides were captured by Leica microscopy with polarised light. The green-thin and red-thick collagen fibre areas of each image were divided by the total subdermal area using ImageProPlus software.

Quantification of vessels using image J

The images were analysed using Image J software (v 1.51m9; National Institutes of Health, USA). Measurements were made in mm, after setting a scale with the reference ruler in the images. Treated target areas (20 $\times$ 20 mm) were selected and duplicated. All vessel lengths inside these areas were individually measured using the “straight segmented” tool of the software. Each vessel measured received a code number and the individual lengths were transferred to an Excel sheet, and organised according to groups. The reduction percentage comparing pre-treatment and six days later was used for statistical analysis. A vascular surgeon, blinded to the group identification, performed all vessel measurements.

Statistical analysis

One way analysis of variance (ANOVA) was performed to identify the differences in vessel length between groups. In addition, the post hoc Bonferroni correction test was used to compare all sub-groups. To analyse the thermographic data, the difference of temperature was calculated between the measurement after the procedure and six days later, always in comparison with the initial temperature. From these differences, two way ANOVA with repeated measures was used and also a post hoc Bonferroni correction test was applied. Data were analysed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and OriginPro 8.1 SR3 (OriginLab Corporation, USA) was used for the statistical analysis, with the significance level set at 5% ($p < .05$).

RESULTS

Qualitative analysis

Rabbit fur re-grew after one day of the experiment, which hindered the quantitative analysis of both white light and thermal images. So, the image analysis was performed immediately after and six days after the procedure.

An increase in both central and peripheral vessel diameters was observed immediately after glucose application in qualitative observation. Sclerosis and disappearance of small vessels was not clearly observed in the images six days after injection of the hypertonic solution.

With PDT application, photographic images showed a net reduction of small vessels in the treated region. There was a reduction of small vessels, which is clear in the images of Fig. 1, and a process of inflammation in the larger vessels. Also observed after PDT was the appearance of a permanent region of damage in the area of treatment.

On analysis of images from control groups, there were no significant qualitative variations in vessel behaviour with white light.

Quantification of vessel effect

Quantification of the vessels was performed with analysis of the images using ImageJ software (Fig. 1).

The results show an average 26% reduction in vessel length in Group 4, significantly greater than in the other groups (Group 3, 2.4%; Group 1, .4%; Group 2, 0). The p value for statistical analysis was $<.001$ with ANOVA test. The Bonferroni test showed a significant difference at the .05 level for all comparisons with Group 4. Fig. 2 shows the percentage reduction and variation in these values. From analysis of Fig. 1, it appears that the reduction was very similar in Groups 1—3, with all close to an imperceptible reduction. However, the quantitative analysis in Fig. 2 shows that there was a small reduction of vessels in Group 3, with Group 4 showing a significant reduction.

Thermal image analysis

Fig. 3 shows typical thermal rabbit ear images during the procedure using PS and light control, glucose, and PDT treatments. In the glucose images, after six days, the temperature returned to normal and the central vessel continued with increased diameter. This behaviour was consistent with the observations from white light images and vessels lengths. In the PDT images, there was a temperature decrease over time. After six days, the central vessel and smaller calibre vessels were not clearly detected in the thermal images, suggesting vessel reduction and lower blood perfusion.[13]

Fig. 4 shows the relative temperature variation in ears during the two treatments performed (glucose and PDT). In both cases, immediately after the procedure, the temperature of the region increased, probably because of an inflammatory process resulting in increased blood supply. After six days, the overall temperature increased in Group 3, but decreased in Group 4, indicating less blood supply to the region under investigation. Statistical analysis with ANOVA was performed for all groups and the p values obtained immediately after treatment and six days later were $<.05$. However, the Bonferroni test showed no significant difference in the comparisons of almost all groups.

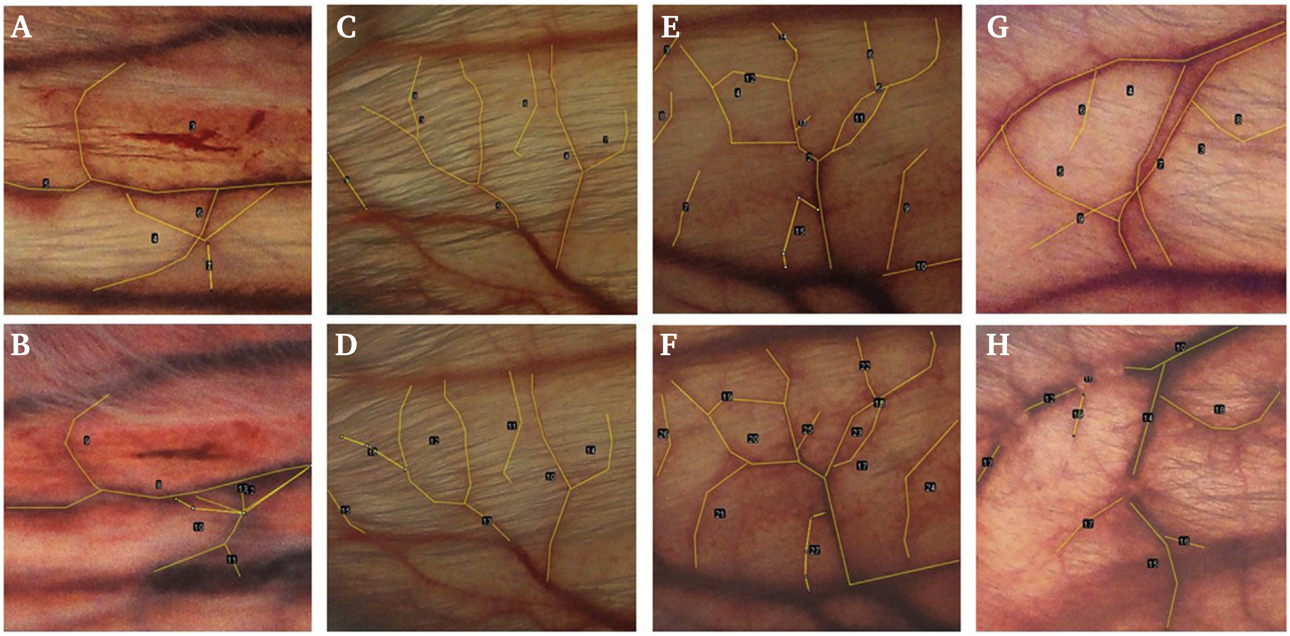

Figure 1. Image of vessels before (top row: A, C, E, G) and after (bottom row: B, D, F, H) all procedures. The yellow lines correspond to the method of vessel length quantification. (A, B) Only injection of photosensitiser (before and after); (C, D) only light (before and after); (E, F) glucose 75% injection (before and after); (G, H) photodynamic therapy (before and after).

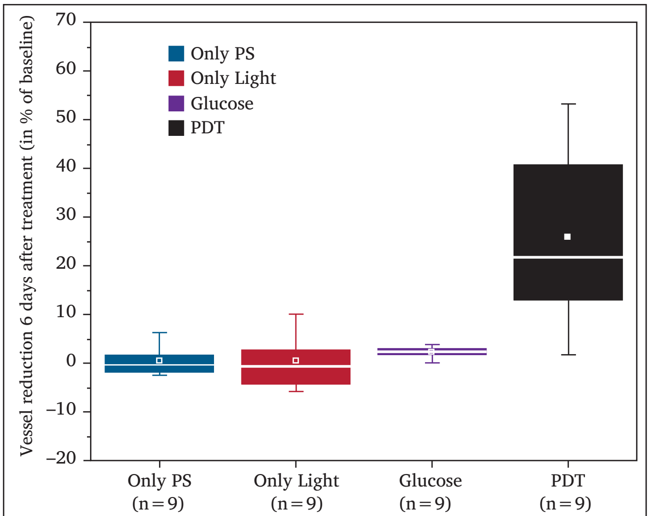

Figure 2. Boxplot graph showing reduction of vessels based on the scores after six days and compared with the baseline images ($p < .001$ for PDT group). PDT = photodynamic therapy; PS = photosensitiser.

With thermal analysis there was no variation in the injection only PS group (Group 1) while in Group 2, with only light, there was a temperature increase immediately after the procedure and six days later this returned to normal. This was an expected increase in temperature because of the high light absorption of blood.

Histological and histochemical analysis

Histological study of the ears by staining with HE and picrosirius red showed some peculiarities as, for example, the presence of granulation in three samples from the Group 3. However, to ensure the integrity of normal tissue, there must be no significant changes in histology, as was observed in the analysis of all groups without major differences between them.

Among characteristics analysed, subcutaneous fibrosis, architectural distortion, hyperkeratosis, and viable vessels were found consistently in all groups (Fig. 5). Histological necrosis, ulceration and haematoma were seen only in one case from Group 4 (Fig. 6).

The observation of the collagen fibres using the picrosirius red method allowed for differentiation of collagen type I - thick fibres (orange-yellowish birefringence to orange and red) and type III - thin fibres (green or yellow-green birefringence). The distribution of collagen fibres in the ears is shown in Fig. 7. The treatment reduced the green area compared with other groups (Fig. 7D); however, it increased the red one (Fig. 7B). The first indicates thin fibres and the second the thick fibres, including types III and I collagen, respectively.

DISCUSSION

Rabbit ear is a commonly used experimental model for vascular treatment.[14,15] However, the small vascular net seen between central artery and collecting marginal vein is actually arteriovenous communications, used physiologically for the animals' temperature control.[16] These vessels have high blood flow, which makes their sclerosis a challenge in some therapies. The marginal vein is easier as a sclerosis target because of its lower blood flow and small calibre (<1 mm), which resembles a reticular vein in humans.[17] This study concentrated on such high flow vessels.

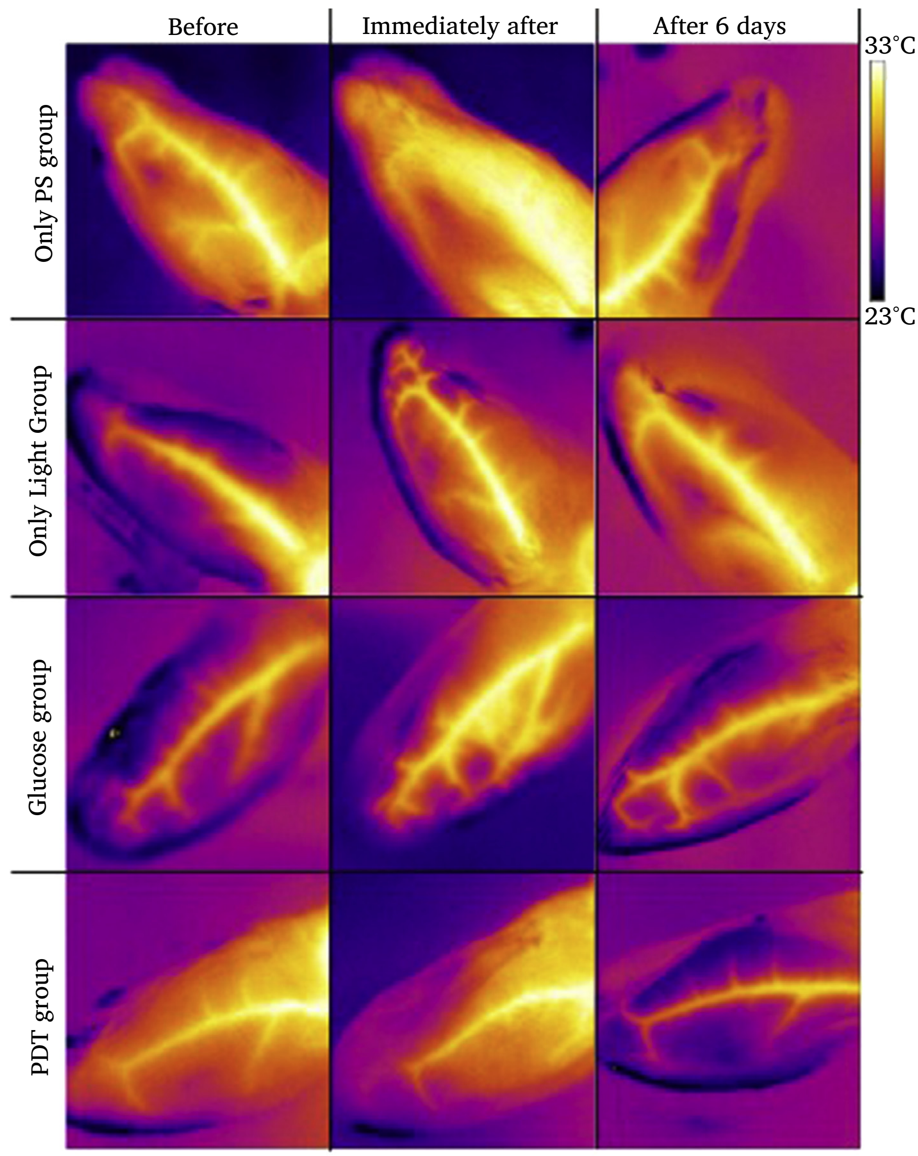

Figure 3. Thermal images for photosensitiser, light, glucose, and photodynamic therapy groups. Examples with the characteristic behaviour before, immediately after, and showing the vessels present after six days of treatment. PDT = photodynamic therapy; PS = photosensitiser.

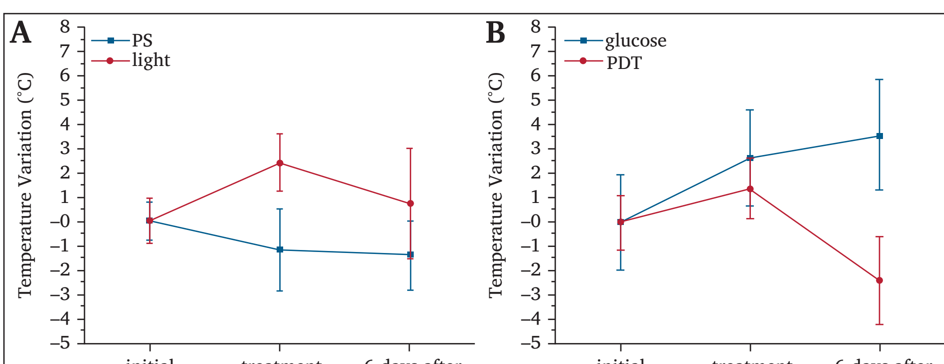

Figure 4. Average temperature variation before, immediately after the treatment, and after six days: (A) calculated for photosensitiser and light control groups and (B) calculated for glucose and photodynamic therapy groups. PDT = photodynamic therapy; PS = photosensitiser.

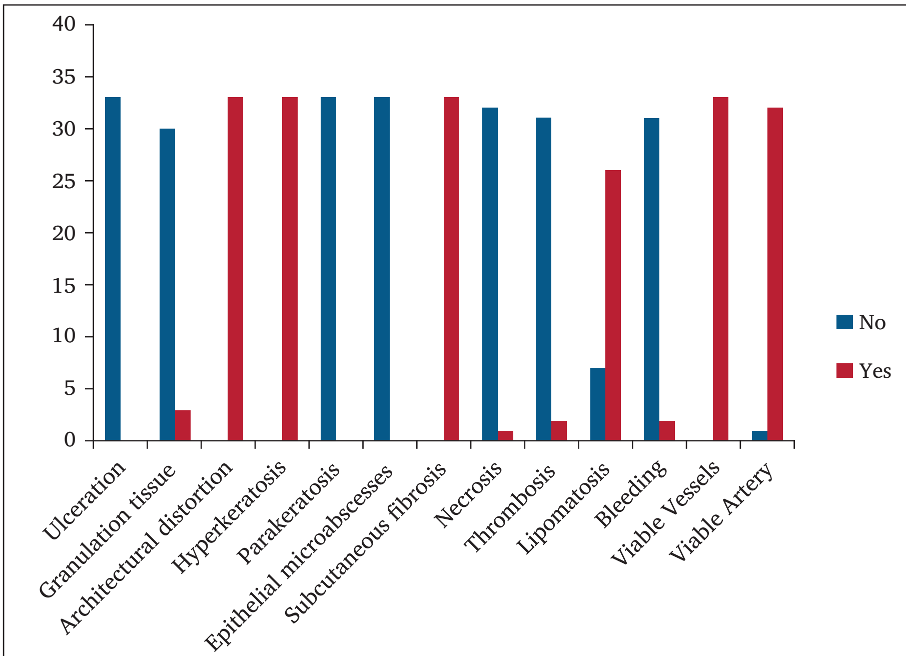

Figure 5. Average histological scores (as defined in Table 1) for different histological processes.

Sclerosing solutions often used in clinical application have osmotic, detergent, or irritating chemical effects. Hypertonic solutions, which are a standard treatment in elimination of small vessels, promote the dehydration of endothelial cells leading to their destruction by osmosis.[18] This effect occurs slowly without serious inflammation, but has an efficiency of about 55% according to the literature.[19]

Conventional therapies, although widely applied in clinical practice, are not sufficient to eliminate all kind of vessels and there is room for further improvement in efficiency. As observed in a qualitative analysis, the sclerosis effect of glucose injection was not marked and, in fact, increased the size of the small vessel network.

In this study, PS and glucose were injected through the central ear artery until the solution reached the entire ear. This made it easier to see propagation of glucose in small vessels, displacing the blood inside them. For Photogem, which has a dark colour and low viscosity, confirmation of injection is slightly more difficult. It was assumed that blocking venous return, the PS agent was in the illuminated area during PDT therapy as observed with fluorescence analysis in a pilot study, done to define the parameters used in this methodology.

The visual appearance of vessels is difficult to quantify because this is a subjective observation and there is some discrepancy between observers. Vessel length measurement using Image J software appears to be a better method because it is an objective measurement and has been used successfully in previous studies.[20] The quantification of vessels in the control groups (Groups 1 and 2) showed no alterations, as expected, and indicated that the light and PS parameters did not cause any damage. The PDT group (Group 4) showed an average reduction of 26% in vessels, reached until 50% (Fig. 2). When compared with the glucose group (Group 3), which presented an average 2% decrease, this result indicates that PDT is much more efficient than glucose.

Thermography is an interesting approach for vessel quantification because skin temperature is related to metabolic rate and blood perfusion in the tissue.[13] Blood perfusion transfers convected heat carried by the blood through the capillaries present in tissue, which is proportional to the temperature difference between arterial blood entering the tissue and venous blood leaving the tissue.[21] Thus, the increased blood perfusion significantly affects the temperature of the tissue, causing heating or cooling.[22] The temperature of the surface tissue also can be affected by alterations in the blood vessels, such as vessel density and blood flow in the area.[13] Skin temperature was also correlated with vessel length determinations. Even showing no significant difference for all groups using the Bonferroni correction test, this test showed a huge difference six days after the treatment comparing Groups 3 and 4, which is important for the future of PDT application.

Although the mechanisms of PDT applied to endothelial cells are not completely understood, there is evidence of major endothelial cells sensitivity to photodynamic damage when compared with other cell lines, such as tumour cells.[5]

In an overview of qualitative analysis of vessels, quantification of images with vessel length and thermography, it is

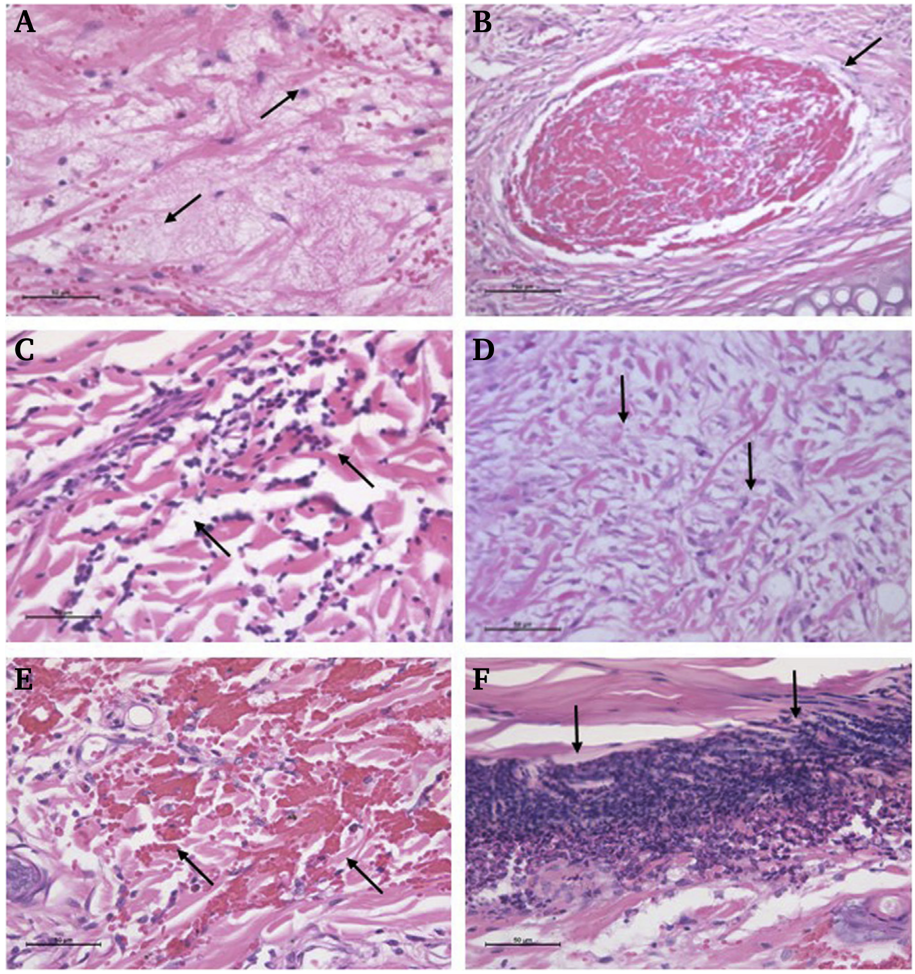

Figure 6. Histological study of the ears by haematoxylin eosin 6 days after telangiectasia treatment. Each image corresponds to a different cell process, indicated by arrows, as follows: (A) histological necrosis (photodynamic therapy, Group 4); (B) thrombosis (Group 4); (C) inflammation process (only light, Group 2); (D) granulation process (glucose, Group 3); (E) haematoma (Group 4); (F) inflammatory process with necrosis and ulceration (Group 4).

possible to infer that the results are favourable to PDT. However, analyses of the results do not indicate complete vascular sclerosis. There are also several parameters to be analysed, such as the best incubation time, route for PS injection, light energy adjustments, and time of application of PS in PDT treatment. However, this to the authors' knowledge, is one of the first publications on PDT in sclerosis of the CEAP 1 vein and the results, although promising, suggest that more PDT studies are needed to improve all parameters.

Histology showed general subcutaneous inflammation, followed by fibrosis. Few cases of necrosis and ulceration were found, and only after PDT treatment. Perhaps in these few examples PDT energy was above safety limits, although within the same level of other tissue PDT applications. Further studies dealing with several levels of energy might shed some light on this controversy. Subcutaneous fibrosis was found in all groups (Fig. 5). Curiously, PDT stimulated more thick fibre proliferation than glucose as seen with picrosirius red staining (Fig. 7). The representative pattern of fibre deposition is demonstrated in the literature,[14] and in fact, this observation was made in a previous study by the present study group, as part of a doctoral thesis[23] (these specific results have not been published in a scientific journal). It is not known why collagen proliferation was stimulated in this experimental set up and why thick collagen fibres increased in PDT treatment.

In this study, the injection was performed in the terminal portion of the artery with the purpose of sclerosing the small communicating branches of the artery with the vein, in an attempt to simulate telangiectasia. However, it is important to consider that this model is not ideal, because the rabbit ear is also the organ responsible for thermal regulation and, therefore, these vessels can dilate themselves at high temperature. Hence, all animals in the lab were maintained under controlled temperature conditions. However, the rabbit ear does not represent exactly the lower limb of the human and it is not possible to directly

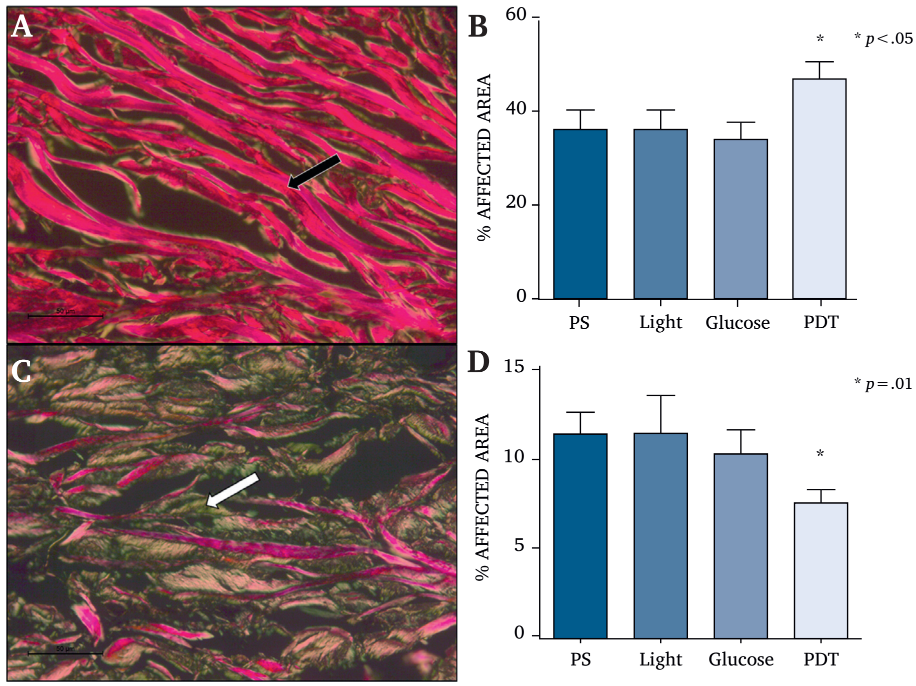

Figure 7. Picrosirius red staining and collagen percentage graphic results 6 days after therapy. (A) Photomicrography of collagen type I thick fibres (coloured in red and indicated by the black arrow); (B) histomorphometric data of collagen I in percentage of affected area; (C) photomicrography of collagen type III thin fibres (coloured in green and indicated by the white arrow); (D) histomorphometric data of collagen III in percentage of affected area. Magnification 400 × . Scale bar, 50 μm. PDT = photodynamic therapy; PS = photosensitiser.

transpose these results for a clinical trial, but they can be used as indicative of the efficacy of PDT.

In conclusion, PDT showed promising results in the sclerosis of small high flow vessels in a rabbit ear model, which were a challenge for glucose sclerosis. The safety of the PDT procedure seemed adequate, as only rare histological alterations were found in the study group. However, further studies dealing with light energy intensity and exposure time, and PS delivery are necessary to find optimum settings for this treatment. In particular, application could be considered to a series of sections, focusing more on vessels with better selectivity, and preserving surrounding tissues.

FUNDING

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CONFLICT OF INTEREST

None.

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