Dose-Related Structural Effects of Photodynamic Therapy on Rabbit Choroidal Structure

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Keywords
Photodynamic therapy · Verteporfin · Dose-dependent effects · Choroidal structure · Central serous chorioretinopathy

Abstract
Introduction: Photodynamic therapy with verteporfin (vPDT) has been shown to be effective against central serous chorioretinopathy (CSC) and was the preferred therapeutic for CSC treatment. However, alterations in choroidal structure after PDT were reported, and these effects were dose-dependent. This study aimed to compare the changes in choroidal structure after PDT with different doses of verteporfin in rabbits and may provide individualized therapeutic guidance for patients who failed to respond to initial half-dose vPDT.

Methods: The full dose of verteporfin used in CSC was 6 mg/m\textsuperscript{2}, which was used in patients with neovascular age-related macular degeneration. Laser fluence was 50 J/cm\textsuperscript{2} (irradiance, 600 mW/cm\textsuperscript{2}, 83 s). There were 4 different dose groups in this study (100%, 70%, 50%, and 30%). The alterations were examined at 1 day, 1 week, and 1 month after vPDT using color fundus imaging, indocyanine green angiography, and histopathology analysis.

Results: Various degrees of choroidal alterations were demonstrated at different dose groups. Examinations on day 1 showed that gradually reduced verteporfin dose tended to decrease phototoxic reactions to the choroid in terms of the number of occlusion vessels and area of the lesion. After 1 month, choroid vessel alteration persisted in high-dose groups (100% and 70%); nevertheless, alterations of low-dose groups (50% and 30%) returned to normal.

Conclusions: vPDT can induce phototoxic reactions of the choroid, high dose causes permanent change, and low dose causes recoverable change. The dose-dependent alterations need to be considered for the individual therapeutic plan according to the situation of a patient with CSC.

Introduction
Central serous chorioretinopathy (CSC) is an idiopathic chorioretinopathy that is characterized by serous retinal detachment involving mainly the macular area and accompanied by leakage of altered retinal pigment epithelium (RPE) [1, 2]. Most acute CSC often resolves spontaneously within a few months; however, in some patients,
it may progress to chronic CSC, which can cause photoreceptor degeneration and RPE atrophy, resulting in irreversible anatomical and functional damage [3]. Recently, the understanding of the pathology causing CSC changed from the RPE level to the choroidal disturbance level [4, 5]. It was thought that photodynamic therapy (PDT) with verteporfin could alter choroidal vasculature structure and perfusion, reduce subretinal fluid (SRF), and improve visual acuity for both acute and chronic CSC [6–8].

The dose of verteporfin infusion and laser energy parameters used in CSC were identical to those used in neovascular age-related macular degeneration (nAMD) (6 mg/m², irradiance, 600 mW/cm², 83 s) [9, 10]. It is generally accepted standard therapy regimen in CSC treatment. Although PDT with conventional verteporfin dose yielded favorable effects, post-PDT complications have been reported in patients, such as RPE atrophy, RPE tear, secondary choroidal neovascularization, and choroidal ischemia [11–13]. As a result, many studies on modified PDT protocols have been evaluated, including lowering verteporfin doses, decreasing laser power, decreasing laser treatment times, or shortening the treatment interval [14–17]. Some studies showed that 50%-dose PDT has favorable results compared with full-dose PDT [14, 18, 19], but there remain failed cases after the initial half-dose PDT. Data on the treatment regimen for patients without response to initial half-dose PDT are insufficient due to the paucity of studies. It has been verified that PDT retreatment for patients with recurrence after failure of initial PDT is effective [20, 21]. The optimum dose for retreatment should be established; therefore, experimental evidence is needed to clarify the impact of multiple drug applications on tissue construct.

Nevertheless, few animal or clinical studies have compared the photochemical reactions of choroid vessels caused by PDT using different doses of verteporfin. This study aims to evaluate choroid vessel structure changes caused by PDT using different doses of verteporfin contributing to treatment response on choroidal vessels in rabbits. The results of this animal study are crucial for not only determining therapeutic effects but also preventing future complications in individualized CSC treatment.

**Materials and Methods**

**Animals**

Adult Chinchilla bastard rabbits (*Oryctolagus cuniculus*, standard Chinchilla) of either sex weighing 2.5–3.0 kg were used for the experiments (obtained from Beijing TianTan Biological Products Co., Ltd., Beijing, China). Rabbits were maintained at the Animal Laboratories of the Peking University People’s Hospital. Animals were used in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University People’s Hospital (permit No. 2014-17).

A total of 33 rabbits were included in this study. They were randomly divided into 7 groups: normal control (3 rabbits), verteporfin only (3 rabbits), laser only (3 rabbits), 100% dose (6 rabbits), 70% dose (6 rabbits), 50% dose (6 rabbits), and 30% dose (6 rabbits). Rabbits were anesthetized using 3% isoflurane inhalation via a facemask. Pupils were dilated with 1% atropine sulfate ophthalmic gel (Xingqi Pharmaceutical Co., Ltd., Shenyang, China), 0.5% tropicamide, and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan). After PDT, rabbits were kept under dark conditions for 3 days to prevent light damage. Then, the animals were euthanized with sodium pentobarbital overdose, and the eyes were enucleated for analysis.

**PDT Procedures**

The currently accepted standard dose of verteporfin used in CSC was identical to the dose used in neovascular age-related macular degeneration patients (6 mg/m²) [9, 10]. Verteporfin (Visudyne, Novartis, AG, Bulach, Switzerland) was used according to the manufacturer’s instructions at a concentration of 2 mg/mL and dissolved in 7 mL of sterile water; the bottle was protected from light and used within 2 h. The 100% dose of verteporfin used in rabbits was 0.43 mg/kg [22], which was extrapolated from the recommended dose in humans of 6 mg/m². Moreover, 70% dose was 0.301 mg/kg, 50% dose was 0.215 mg/kg, and 30% dose was 0.129 mg/kg.

For PDT lesions, a diode laser at 689 nm with a slit-lamp delivery system (Ocal Photoactivator, Lumenis Inc, Santa Clara, CA, USA) was used. Marginal ear infusion of verteporfin was performed for 10 min, followed by laser delivery at 15 min from the start of infusion. The effect of laser illumination was evaluated at a fluence of 50 J/cm² (irradiance, 600 mW/cm², 83 s). A factor of 0.66 for the rabbit eye was used [23], and the diameter of the laser spot was 2,500 μm. Two laser spots were placed at a distance of 1/2 papillary diameter from the inferior optic disc margin. All rabbits received PDT in 1 eye, and the other eye did not receive any treatment. To ensure consistency, PDT was performed by an experienced ophthalmologist.

Fig. 1. Color fundus image. a1 Control group showed normal fundus. a2 Verteporfin-only group. a3 Laser-only group showed normal fundus 1 day after treatment. b1–3 Fundus photographs of the 100% dose group. c1–3 Fundus photographs of the 70% dose group. d1–3 Fundus photographs of the 50% dose group. e1–3 Fundus photographs of the 30% dose group. Retinal detachment and transient SRF were noted 1 day after treatment in all treatment groups (b1–e1); 1 week after treatment, the 100% dose and 70% dose groups still had retinal detachment and SRF (b2, c2); retinal detachment and SRF disappeared in the 50% dose and 30% dose groups (d2, e2); 1 month after treatment, RPE mottling was observed in the 100% dose and 70% dose groups (b3, c3); retinas of the 50% dose and 30% dose groups returned to normal (d3, e3). SRF, subretinal fluid.
Dose-Related Effects of Photodynamic Therapy on Choroidal Structure

Control Verteporfin only Laser only

1 day 1 week 1 month

100% dose

70% dose

50% dose

30% dose

a1 a2 a3

b1 b2 b3

c1 c2 c3

d1 d2 d3

e1 e2 e3
W of treatment, 100% dose and 70% dose groups still to the laser irradiation spot appeared (Fig. 1b1–e1). After dose group, only slight retinal whitening corresponding decreased from 100% dose group to 50% dose group; in 30% Retinal detachment and transient SRF were gradually de-

responses were demonstrated in different dose groups. (Fig. 1a2, a3); however, various degrees of chorioretinal (Fig. 1a1). After PDT, choroid vessels showed reperfusion, but existed in small areas of hypofluorescence; ICG angiography of 70% dose group (c1–

d1–3 ICG angiography image. a1 Control group showed normal ICG angiography image. a2 Verteporfin-only group. a3 Laser-only group exhibited normal chorioidal vessel appearance without leakage or occlusion. b1–3 ICG angiography of the 100% dose group. At 1 day after PDT, hyperfluorescence corresponding to the laser spot size and hypofluorescent patch inside the laser lesion were noted; at 1 week after PDT, hyperfluorescent still in the center of the laser spots, with a hyperfluorescent ring in the periphery. At 1 month after PDT, choroid vessels showed reperfusion, but existed in small areas of hypofluorescence; ICG angiography of 70% dose group (c1–

b1–3). Angiography findings were consistent with 100% dose group. b1–3 ICG angiography of 50% dose group. At 1 day after PDT, the choroid presented hyperfluorescence ring, and hypofluorescent patch area inside the laser lesion was noted. At 1 week after PDT, hypofluorescence was observed, while the hyperfluorescence ring decreased. At 1 month after PDT, choroid vessels were normal; ICG angiography of the 30% dose group (e1–3). At 1 day after PDT, angiography findings were consistent with those of the 50% dose group. At 1 week after PDT, no alteration of choroid vessels was observed. ICG, indocyanine green; PDT, photodynamic therapy. (For figure see next page.)
Dose-Related Effects of Photodynamic Therapy on Choroidal Structure

Control  Verteporfin only  Laser only

1 day 1 week 1 month

100% dose

70% dose

50% dose

30% dose

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Histopathologic Findings

Histologic examination of laser-only group and verteporfin-only group showed a normal pattern without any observable pathologic change in the retina and choroid tissue at 1 D after treatment (Fig. 3a–c, Fig. 4a–c). Light microscopy of H-E staining and electron microscopy showed a pattern of milder photochemical reaction to the choroid and retina structures with decreasing verteporfin dose levels. The histological changes were mainly the same between the first and second irradiation spots in the same eye. H-E staining sections of 100% dose and 70% dose groups revealed not only thrombosis of medium and large size choroidal vessels but also destruction of the RPE and OS (outer segment) and large amount of subretinal exudation 1 D after irradiation. On day 7 after treatment, choroidal vessel thromboses nearly resolved, a small amount of exudation remained, and RPE cell layer discontinuity did not recover. At 1 M, the blockage of choroidal vessels almost disappeared, choroid fibrous proliferation and exudation resolved, and there was thinning of the outer nuclear layer (Fig. 3d–e).

In the 50% dose group, 1 D after PDT, distended choroidal vessels and very few obstructed choriocapillaries can be detected, and modest disruption of RPE, photoreceptor cell layer, and intermediate subretinal exudation were observed. At 1 W after PDT, dilatation of the choroid vessels was observed, and RPE cell discontinuity did not recover. After 1 M, choroid and retina structures returned to normal (Fig. 3f).

Histological damage in the 30% dose group was milder than that in the 50% dose group. Small but visible exudation, distended choroidal vessels, and fewer obstructed choriocapillaries were detected in H-E staining section on day 1 after PDT. After 1 W, dilatation of the choroid vessels was observed, and other tissues recovered to normal. At 1 M, both choroid and retina structure returned to normal (Fig. 3g).

On electron microscopy, intact choriocapillary microstructure was detected in the verteporfin-only and laser-only groups compared with the control group (Fig. 4a–c). All PDT with verteporfin groups showed damage to choriocapillary microstructure 1 D after PDT. Bruch’s membrane showed discontinuity, and choriocapillary endothelial cell displayed severe swelling. In the vessel lumens, monocytes and hemolyzed red blood cells were observed (Fig. 4d–g). The degree of severity differed among the 4 groups. The 100% dose group (Fig. 4d) and the 70% dose group (Fig. 4e) had more occluded vessels, and the response degree was milder in the 50% dose group (Fig. 4f) and the 30% dose group (Fig. 4g). The 30% dose group also showed less severe condition than the 50% dose group. At 1 W after PDT, the choriocapillary in 100% dose and 70% dose groups showed rough basal lamina, and the fibrin was visible in the vascular lumina and the surrounding tissue. The choriocapillary microstructure of 30% dose and 50% dose groups almost returned to normal. On 1 M after laser irradiation, abnormalities of the vascular lumen, basal lamina, and endothelial cell were still prominent in 100% dose group and 70% dose groups, and the basal lamina was still lightly rough and had occasional discontinuity. The other 2 groups showed a normal vascular microstructure.

Discussion

PDT was originally used as a clinical therapy for malignant tumors. In 2003, it was proposed for patients with CSC. Previous studies have demonstrated beneficial visual outcomes in most patients [6, 24, 25]. One limitation of the current PDT treatment was the damage to normal structures. Additional adverse effects to the normal retina, RPE, and choroid caused by PDT are dose-dependent [26, 27]. To prevent adverse effects, half-dose or low-fluence PDT has been studied in the treatment of CSC. A retrospective study conducted by Nicolo et al. [28] revealed that, although half-dose PDT and half-fluence PDT had equal visual improvement and safety in long-term follow-up, half-dose PDT had the advantage of a more rapid reabsorption of the fluid and lower recurrence rate. Decreased fluence PDT might cause more significant inflammatory reaction or increase collateral damage to the normal tissue. To improve treatment safety in CSC, half-dose PDT was proposed. The resolution rates of the SRF after half-dose PDT vary from 60% to 95% [29–31]. Our previous study evaluated a range of verteporfin doses from 10% to 70% and found that 30% was the lowest effective dose, while 50% was better than a 30% with SRF completely resolved in 95% of patients [32].

However, recurrence of SRF occurred in some patients (10–20%) after initial 50%-dose PDT [33]. These patients required re-treatment. It is believed that patients with persistent SRF for at least 3 months since the previous PDT should benefit from re-treatment, especially if there has been a positive response [34]. To the best of our knowledge, presently, the re-treatment strategy for eyes with persistent SRF did not have uniform standard. The application of higher dose vPDT in patients requiring re-treatment leads to a better therapeutic efficacy but may
Fig. 3. H-E staining of rabbit choroid vessels. **a** Control group: normal rabbit retina and choroid. **b** Verteporfin-only group. **c** Laser-only group showed a normal pattern without any pathologic change. **d** In the 100% dose group, at 1 day after PDT, vessel closure of choriocapillaries and deeper choroidal vessels were observable (arrows), OS layer condensed, RPE destructed, and SRF (*) was damaged. At 1 week after PDT, choriocapillaries thromboses nearly vanished, RPE cell layer showed discontinuity, and a small amount of exudation remained. At 1 month after PDT, choriocapillary occlusion disappeared, and choroid fibrous proliferation and exudation resolved. **e** In the 70% dose group, the same was found. **f** In the 50% dose group, at 1 day after PDT, distended choroidal vessels, few obstructed choriocapillaries (arrows), and mild SRF (*) were observed. At 1 week after PDT, dilatation of the choroid vessels was observed, and RPE cells discontinuity did not recover. After 1 month, choroid and retina structure returned to normal. **g** The same was found in 30% dose group, but the damage was milder than that in the 50% dose group. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment; RPE, retinal pigment epithelium; SRF, subretinal fluid; H-E, hematoxylin and eosin; PDT, photodynamic therapy; arrow, occluded choroid vessel; *, SRF; scale bar, 50 μm.
also result in potential adverse events. It is important to minimize potential retinal toxicity during PDT while maintaining the treatment effects. The complex structural changes of the chorioretinal anatomy that limits in vivo imaging, such as optical coherence tomography and ICG angiography, indicated that much of our understanding of this critical structure has originated from histopathological analysis.

Recently, the new imaging technique of optical coherence tomography angiography (OCTA) has improved the visualization of the chorioretinal vascular structures and microcirculation in vivo [35]. Demircan et al. demonstrated choriocapillaris alterations using OCTA after half-fluence PDT at 3 days and 30 days following therapy. They concluded that there was a markedly decreased choriocapillary flow limited to the site of the PDT spot in the very early period following PDT in eyes with CSC, and the choriocapillary perfusion returned to normal at day 30 [36]. In the study by Alovisi et al. [37] measured by OCTA scan, half-dose PDT seems to produce short-term (1 week after PDT) changes on the luminal component of both the choriocapillary and choroid, which returned to normal status at 1 month from treatment. Although OCTA has proven to be a valuable tool for the depth-resolved evaluation of the retinal and choroidal structures, the structural changes of choriocapillary and choroid tissue after PDT are still unclear.

Normal choriocapillary and choroid structure changes after PDT are unavailable in humans. Our observation represents a novel finding in an animal model. Even 30% dose vPDT yielded minimal choroid change, as choroidal hyperfluorescence corresponding to the laser spot size and a hypofluorescent patch area inside the laser lesion on fundus photography and ICG angiography were observed. Histopathology showed distended choroidal vessels after treatment. Under different levels of verteporfin dose, the edges of choriocapillary alteration are different. Therefore, it might be speculated that with low verteporfin dose, the effect of PDT is just small endothelial lesions (angiographic hyperfluorescence without vessel occlusion) rather than vessel clotting, so the initiation of a 50% dose might be too low to have a significant effect in some patients. With increasing verteporfin dose, the alteration in choroid vessel increased, may further promote vascular occlusion or thrombosis (angiographic hypofluorescence), and plays a positive role in patients requiring re-treatment. However, it may potentially cause side effects when the verteporfin dose was extremely high.

A human clinical trial reported full-dose vPDT, revealed choroidal hypoperfusion with choriocapillary destruction at 2 years of observation [39], and suggested permanent closure of part of choroidal vasculature. Some clinical studies confirmed the 30% dose seemed to be safe and effective in CSC treatment; therefore, successful rate in the 30% dose was < that in the 50% dose, and recurrence rate in the 30% dose group was > the 50% dose group [32, 40, 41]. Thus, in our clinical work, patients with failure to initial half-dose treatment required retreatment with the same dose or gradually increased dose and 70% dose groups choriocapillary showed rough basal lamina, and cell debris was visible in the vessel lumen. Choriocapillary microstructure of the 30% dose and 50% dose groups almost recovers to normal. At 1 month after PDT, abnormalities of vascular lumen and endothelial cell were still prominent in the 100% dose and 70% dose groups. Moreover, the 50% dose and 30% dose groups appeared a normal vascular microstructure. En, endothelial cell; Pe, pericyte; RPE, retinal pigment epithelium; OS, outer segment; PDT, photodynamic therapy; arrowhead, Bruch’s membrane; *, vessel lumen; scale bar, 2 μm.

(For figure see next page.)
verteporfin dose. In the retrospective study of Oh et al. [42], patients with relapse were received additional treatment, all had a good clinical prognosis with a decrease in or a complete absorption of SRF, and none of them had ocular or systemic adverse events. The dose of re-treatment was not reported in their study. However, through histological experiments, we showed that more serious tissue damage occurred in the higher dose group. Observations of the PDT effect on the choroid tissue are valuable for treatment selection in clinical practice. Eyes with persistent SRF or recurrence after initial half-dose PDT, they needed additional re-treatment. It is crucial for patients to select the appropriate re-treatment option. Therefore, we advocate starting with low dose and escalating to full dose based on treatment response in clinical PDT.

Our study has several limitations, including small sample size and short-term follow-up period. To avoid more sacrifice, we used 6 rabbits for each group. Moreover, the adverse effect of PDT on choroidoretinal function should be observed after treatment of different doses.

In summary, PDT with verteporfin used clinically in the treatment of CSC induces injury of the physiological choroid, high dose may cause permanent alteration, and low dose causes slight change. The ophthalmologist should make individualized treatment plans according to patients’ situation, especially for those patients who failed in the initial PDT and required multiple re-treatments. This will be beneficial for patients to reach optimal therapeutic effect and diminish side effects.

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