The Chorioretinal Damage Caused by Different Half Parameters of Photodynamic Therapy in Rabbits

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Abstract

Purpose: To compare the chorioretinal tissue response after different half-strength parameters of photodynamic therapy (PDT) in rabbits.

Methods: The study included 4 groups, and each group contained 4 animals. The full dose served as the control group: verteporfin (4 mg/kg) with 600 mW/cm² irradiance from a diode laser at 689 nm applied to the retina for 8 s. One parameter was changed to half-strength in the other 3 groups. The HLaser group received half-strength laser irradiance. The HTime group was exposed to photosensitization for half the time, and the HDose group received half the drug dose. Six laser spots were generated in each of the eyes of every rabbit and documented graphically. The lesions were examined on days 1, 7, and 42 after PDT treatment using color fundus imaging, fluorescein angiography (FA), and histopathology analysis.

Results: PDT treatment in rabbits caused chorioretinal damage in all 4 groups. FA on day 1 showed that the use of half the laser irradiance, half the drug dose, or half the photosensitizing time tended to decrease the damage to the chorioretinal tissue in terms of the number of occlusions and the area of occlusion, but only the results from half the laser irradiance were significantly different. In addition, the HLaser and HDose groups showed significantly less apoptosis by TUNEL staining on day 1.

Conclusions: Among these PDT parameters, decreasing the laser irradiance by half showed the greatest decrease in chorioretinal damage in an experimental animal model.

Introduction

PHOTODYNAMIC THERAPY (PDT) has been an option for the treatment of choroidal neovascularization (CNV) due to age-related macular degeneration (AMD) and pathological myopia because it has the potential to minimize damage to the surrounding viable retinal tissue compared with laser photocoagulation.^{1–3} At the beginning of standard PDT, the patients receive a verteporfin injection at a concentration of 6 mg/m² of body surface area over a 10-min period. Five minutes after completion of the infusion, patients undergo PDT with a light fluence of 50 J/cm². Verteporfin, a benzoporphyrin derivative, is a photosensitizer for PDT. When stimulated by nonthermal red light with a wavelength of 693 nm in the presence of oxygen, verteporfin produces reactive singlet oxygen, resulting in local damage to the endothelium and blockage of blood vessels.^{4,5} Husain

et al. reported that digital angiography can be used to demonstrate that verteporfin can localize around the CNV after intravenous injection.⁶ In the next step of PDT, a nonthermal laser is applied focally to the photosensitized drug around the CNV to produce photochemical reactions and lead to new vessel occlusion. However, severe visual loss and collateral damage after PDT are reported, and these effects are dose-dependent.^{7–9} Therefore, PDT became a second-line treatment option for CNV after the introduction of anti-VEGF antibodies.¹⁰ However, PDT has become popular and remains useful in treating non-CNV maculopathy, such as chronic central serous chorioretinopathy (CSC) and polypoidal choroidal vasculopathy (PCV).^{11–13}

Recently, the treatment of CSC by PDT has yielded beneficial visual outcomes in the majority of patients.^{11,12,14,15} The mechanism of PDT in treating CSC is thought to be the result of choriocapillaris remodeling, leading to a reduction of

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choroidal exudation and choroidal vascular remodeling instead of vessel occlusion.^{12,14–16} However, complications such as retinal pigment epithelium (RPE) atrophy have been reported after PDT for chronic CSC.¹⁷ Moreover, with the use of mf ERG, clinical and laboratory studies have demonstrated a transient reduction in macular function following PDT.^{18,19} Reducing the dose of verteporfin might minimize the potential retinal damage caused by PDT while at the same time maintaining photodynamic effects on the choroidal vasculature sufficient to treat CSC. The efficacy and safety of using a half dose of verteporfin in the treatment of either acute or chronic CSC have been demonstrated.^{12,13,17,18,20} The safety and efficacy of this half- or reduced-dose concept have also been shown in the treatment of PCV cases.¹³

The adequate dosage of verteporfin in the PDT of CSC or PCV remains an open question, and the damage to the retina caused by PDT is dose-dependent.¹⁴ According to a previous study, even very low fluencies of PDT could damage the choriocapillaris in animals.²¹ Nevertheless, few animal studies or clinical randomized studies have compared the damage caused by PDT using different parameters. Therefore, we aim to study the factors contributing to PDT damage by using a half dose of verteporfin, half-strength laser irradiance, or half the photosensitizing time to determine the influence of these factors on chorioretinal damage in rabbits. The results of this animal study are crucial not only for determining therapeutic effects but also for preventing future complications in CSC or PCV treatment.

Methods

Animals

Rex pigmented male rabbits weighing between 2.0 and 3.0 kg and ~6 months old were used for the experiment. The animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Animals were anesthetized with an intramuscular injection of 0.15 mL/kg of an equal-volume mixture of 2% lidocaine (Xylocaine; Astra) and 50 mg/mL ketamine (Ke-

talar; Parke-Davis). Pupillary dilatation was achieved with a topical application of phenylephrine hydrochloride 2.5% and tropicamide 1%. A Q lens contact lens was placed onto the mydriatic eye using sodium hyaluronidase (Healon; Pharmacia and Upjohn) as a contact gel. After PDT, the animals were kept under dark conditions for 3 days to prevent light damage. The animals were then killed with an intravenous injection of sodium pentobarbital, and the eyes were enucleated for analysis.

PDT procedures

After anesthesia and pupillary dilatation, we injected verteporfin into the ear vein and, 10 min later, performed PDT to simulate the treatment received by clinical patients, as described previously.²¹ A total of 20 pigmented rabbits were included in this study; each group contained 4 animals. The rabbits received a half drug dose, half the laser irradiance, half the photosensitizing time, or the control treatment. The control received the full dose of verteporfin (4 mg/kg) and 600 mW/cm² irradiance from a diode laser at 689 nm applied to the retina for 8 s. The HLaser group received half-strength laser irradiance: verteporfin (4 mg/kg) with 300 mW/cm² irradiance from a diode laser applied to the retina for 8 s. The HTime group was exposed to photosensitization for half the time: verteporfin (4 mg/kg) with 600 mW/cm² irradiance from a diode laser applied to the retina for 4 s. The HDose group received a half drug dose: verteporfin (2 mg/kg) with 600 mW/cm² irradiance from a diode laser applied to the retina for 8 s. Six laser lesions were applied to both eyes of every rabbit, in which 3 lesions were placed at the retina superior to the optic disc and the other 3 inferiorly (Fig. 1). These lesions were documented graphically. A conventional diode laser at 689 nm with a slit-lamp delivery system (Zeiss Visulas 690 s and Visulink PDT; Zeiss) was used. To determine the appropriate spot size for the choroidal lesions, a factor of 0.66 for the rabbit eye was initially used and then adjusted before treatment. The retinal spot was 2,500 μ m in diameter. The power output at the slit lamp was confirmed prior to the treatment using a handheld power meter.²¹

FIG. 1. Fluorescein angiography (FA) of rabbits. (A) FA picture of the Control group (the full dosage group). The 6 white spots are the laser lesions. (B) FA picture of the HLaser group (half laser irradiance group). (C) FA picture of the HTime group (half photosensitization time). (D) FA picture of the HDose group (half drug dosage). Note that the FA showed an occlusion area (dark area inside the bright spots) inside the damaged area (bright spots) in (A, C, and D) but not in (B).

To evaluate the effect of the diode laser itself, a separate experiment was performed in 4 rabbits. In 3 rabbits, we applied 600 mW/cm² irradiance from a diode laser at 689 nm to the retina for 8 s without injection of verteporfin. A fourth rabbit did not receive laser treatment, as a laser-naïve control.

Color fundus imaging and fluorescein angiography

The PDT lesions were studied on days 1, 7, and 42 after PDT treatment by color fundus imaging and fluorescein angiography (FA). The anesthetized animals were injected with 0.1 mL/kg of 10% fluorescein sodium (Fluorescite; Alcon) via the ear vein. This enabled the detection of damage to the vessel endothelium cells and the RPE, which results in the pooling of fluorescein in the subretinal space and leads to hyperfluorescence, and the detection of chorioidal vessel closure, which leads to hypofluorescence. Late-phase angiograms were obtained 8 min after injection, and digital fundus images of bilateral eyes were taken within 1 min. The collateral chorioretinal change after PDT was evaluated by FA as described in a previous study. A hypofluorescent area inside the laser lesion indicated vessel occlusion, and a hyperfluorescent area indicated damage to the choriocapillaries but not occlusion.²¹

The fundus imaging system used in our research was adapted from that described by Paques et al.²² Our modified system consisted of a surgical endoscope with a 4-mm outer diameter (27005AA; Karl Storz), a Nikon reflex digital camera [D80 with a 10-million-pixel charge-coupled device (CCD) image sensor], an adaptor (590-70; Karl Storz) to connect the camera to the endoscope, and an AF 85/F1.8 D objective (Nikkor). The light source was a xenon lamp (201315; Karl Storz) connected to the endoscope through a flexible optic fiber. The camera settings were as follows: manual focus, operating mode S (shutter speed priority), shutter speed set at 1/100 s, and automatic white balance setting.

Histopathology and in situ TUNEL

For the histology analysis, eyeballs were harvested and fixed in 4% paraformaldehyde at 4°C for 24 h. The fixed tissues were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. The posterior eye cup was cut from the anterior segment after 10 min and replaced in the fixative. The retinas were then fixed in 2.5% glutaraldehyde, postfixed in Dalton's osmium fixative, dehydrated in alcohol, and embedded in epoxy resin (Epon). Sections were stained with uranyl acetate. Serial sections (1 µm thick) were cut until the center of the lesion was reached.

For apoptotic analysis by *in situ* TUNEL, eyeballs were harvested and marked for orientation, fixed in 4% paraformaldehyde at 4°C for 24 h, embedded in paraffin, and sectioned (5 µm sagittal sections). The sections were dewaxed in xylene and progressively hydrated. The chorioretinal sections from the temporal and nasal quadrants were assayed. The TUNEL method was performed using an apoptosis terminal deoxynucleotidyltransferase (TdT) DNA fragment detection kit (TdT-FragEL; Oncogene) according to the manufacturer's instructions. Briefly, 5 µm thick paraffin sections were deparaffinized in xylene and rehydrated through a graded series of alcohol and distilled water. They

were treated with proteinase K for 15 min at room temperature and washed in distilled water. Endogenous peroxidase was inactivated by incubating the sections with 3% H₂O₂ for 5 min at room temperature and washing in distilled water. The sections were incubated with biotin-16-dUTP, TdT, and 20% 5X cacodylate buffer in a moist chamber for 1 h at 37°C and washed in PBS. They were treated with peroxidase-conjugated streptavidin for 30 min at room temperature and washed with PBS. Diaminobenzidine was used as a chromogen. Counterstaining was performed with methyl green. To compare the levels of apoptosis in different groups, the apoptotic cells were counted from montage images of the chorioretinal cryosection and averaged from 5 serial sections cut from lesions in each eye ($n=4$ eyes in each group). The number of apoptotic cells per 20 rows of retinal cells, as determined and processed statistically from montage images of the chorioretinal cryosection, were counted and compared in the center of the PDT damage area.

Electron microscopy

The rabbits were sacrificed, and the eyes were collected and immediately placed in 4% glutaraldehyde. Then, the specimens were postfixed with 1% osmium tetroxide (Next Chimica), dehydrated, and embedded in Epon (Araldite-502 Embedding; Electron Microscopy Sciences). The retinal sections were obtained, contrasted with uranyl acetate and lead citrate, and analyzed using a Hitachi EM H-7100 electron microscope (Hitachi).²³

Statistical analyses

In this study, data were entered into Excel and analyzed by the Statistical Package for the Social Sciences (SPSS). Although there were 4 groups of 4 rabbits, we analyzed the results of 6 lesions of each eye for a total of 48 lesions in each group. The differences between the 4 groups ($n=48$ lesion in each groups) were evaluated by the Kruskal–Wallis test for analysis of occlusion. Additionally, comparing the apoptosis of cells by TUNEL method was also analyzed by Kruskal–Wallis test. All the results presented as mean $\pm$ SD. A P value <0.05 was considered significant.

Results

All animals were examined by color fundus imaging and FA on day 1 after PDT treatment. Two animals in each group were separately sacrificed on day 1 and day 42 to perform a histological analysis. The color fundus imaging and FA were also performed on days 7 and 42 for 2 animals in each group. The PDT treatment induced faint laser lesions that were detected by the color fundus examination. Moreover, FA demonstrated a hyperfluorescent area inside the laser lesion, representing retinal tissue damage, and a hypofluorescent area inside the laser lesion, representing vessel occlusion and thus suggesting more severe chorioretinal damage, as previously described.²¹ FA on day 1 showed different degrees of damage in different groups; more occluded areas were found in the full-dose group (Fig. 1). The histological and TUNEL examinations showed that the effect of PDT on chorioretinal tissue was homogenous and the margin was well defined. Only the area inside the PDT spots exhibited apoptosis on day 1, as shown by TUNEL staining. Retinal damage caused a disorganized retina on day 42, as

FIG. 2. Histological staining and TUNEL showed that the effect of photodynamic therapy (PDT) on chorioretinal tissue was homogenous and that the margin was well defined. **(A)** Apoptosis on day 1 is shown by TUNEL. Inside the PDT spots, the margin is indicated by a red arrow. **(B)** Retinal damage causing a disorganized retina on day 42 is shown by histology. The margin is indicated by a red arrow. Note that the outer nuclei are completely absent. These 2 images were montaged by computer software. The cubic area in **(A)** is further magnified and shown in **(A1)**, and the cubic area in **(B)** is further magnified and shown in **(B1)**.

shown by the histological analysis (Fig. 2). To compare the photodynamic effect of various PDT regimens, the chorioretinal damage was quantified based on the FA findings.²¹ Both the incidence of occlusion among the 6 laser burns per eye and the ratio of occlusion in the damaged area among the 4 groups were analyzed separately.

In the analysis of the incidence of occlusion among the 6 laser burns per eye, the highest incidence was $60\% \pm 2\%$ in Control, and the lowest was $20\% \pm 2\%$ in HLaser. Interestingly, HLaser was the only group whose incidence of occlusion was significantly different from control ($P=0.017$, Kruskal–Wallis test) (Fig. 3A). In the analysis of the ratio of occlusion in the damaged area, the higher the ratio, the more serious the collateral choriocapillary hypoperfusion after PDT. Again, HLaser was the only group that had a significantly lower ratio of damaged area than Control ($3\% \pm 4\%$ vs. $17\% \pm 5\%$, $P=0.043$) (Fig. 3B). Comparing the 4 groups, the FA results showed that HLaser decreased the severity of induced choriocapillary hypoperfusion in terms of the incidence and the ratio of occlusion in the damaged area following PDT treatment.

The apoptotic cells in the laser-damaged area were counted by TUNEL staining in the retinal section 1 day after

PDT (Fig. 4). HLaser had a lower number of apoptotic cells (39.8 ± 12.7 cells/20 rows of apoptotic cells) than Control (131.5 ± 6.6 cells/20 rows of apoptotic cells), HTime (65.8 ± 14.9 cells/20 rows of apoptotic cells), and HDose (114.3 ± 9.1 cells/20 rows of apoptotic cells) after analysis. TUNEL showed significantly less apoptosis in HLaser and HDose compared with the Control on day 1 after PDT (Fig. 5).

In the separate experiment on the damage caused by the diode laser, the results showed this nonthermal laser slightly damaged the retinal tissue. Although the RPE and choroidal tissue seemed normal through 6 weeks after laser treatment, damage to the photoreceptors was observed in the rabbits after only diode laser treatment on day 7 and 42. However, this damage to photoreceptors was not obvious on day 1 after laser treatment (Fig. 6).

Discussion

Our results demonstrate that the half-laser irradiance treatment induced the least damage among the tested “halved” PDT treatments. Although decreasing the other PDT parameters, including the drug dose and the photosensitizing time, also tended to reduce the collateral

FIG. 3. Kruskal-Wallis test analyzing the incidence and ratio of occlusion caused by the treatment. Comparing the 4 groups ($n=48$ lesions in each group), the values for HLaser (the half laser irradiance group) were significantly lower than those of the others (A). HLaser was the only group that had a significantly lower ratio of damaged area than Control (full-dose group) (B).

damage, half the laser irradiance was the only treatment that showed significantly less collateral damage to the retinal tissue. This finding implies that we need to consider reducing the damage done to human patients by adjusting the laser irradiance in clinical applications of PDT for non-CNV maculopathy, including CSC and PCV. To avoid more sacrifice, we use 4 rabbits (6 lesions in both eyes) for each group and small number of animals is the limitation of this study. In the past, it was suggested that this nonthermal

diode laser caused no harm to retinal tissue when used alone in PDT. However, this study shows that it produces significant retinal damage in PDT treatment. In a separate animal experiment, electron microscopy (EM) showed that retinal tissue was irritated by a diode laser treatment without verteporfin drug infusion, and this diode laser actually caused retinal damage in rabbits. According to the EM examination, photoreceptor damage occurred after only diode laser treatment on day 7 and 42. Adjusting the laser irradiance in

FIG. 4. The apoptotic cells in the laser-damaged area shown by TUNEL staining in the retinal section. (A) HE stain of a normal rabbit retina. (B) Control: full drug dose. (C) HLaser: half the laser irradiance. (D) HTime: half the photosensitizing time. (E) HDose: half the drug dosage. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

FIG. 5. The TUNEL stain showed significantly less apoptosis in the HLaser group (half the laser irradiance) and the HDose group (half the drug dosage) compared with the Control group (full-dose group).

PDT treatment may play a key role in damage reduction in future clinical applications.

Recently, PDT has not been the first option for the treatment for macular CNV, due to the introduction of anti-VEGF antibodies.²⁴ However, recent publications have shown that the effects of anti-VEGF therapy are limited in PCV cases.²⁵ Laser photocoagulation is a treatment option for both CSC and PCV,²⁶ but it only works for lesions far away from the fovea.²⁷ Additionally, direct thermal laser photocoagulation has the disadvantage of causing RPE damage and, rarely, iatrogenic CNV.^{28,29} Nishijima et al. reported that laser photocoagulation is useful for ICGA-identified feeder vessels, but complete occlusion after the first treatment occurred in 6 of 15 cases.³⁰ In contrast, PDT with verteporfin has been shown to be effective in reducing SRF and improving visual acuity for chronic CSC and PCV.^{11–13} Currently, only the standard PDT parameters that have been thoroughly studied are recommended by the TAP guidelines in the treatment of wet AMD.³¹ However, these standard parameters of PDT that are used to treat CNV appear to damage the physiological choriocapillary layer beyond the irradiated area, with repeated PDT even leading to persistent choriocapillary nonperfusion in most eyes.^{16,32}

The optimal verteporfin parameters for PDT of CSC or PCV, which have pathology different from that of CNV, remains an open question. A previous animal study showed that even very low doses of PDT can damage the choriocapillaris.²⁴ Additionally, the damages and complications caused by PDT are dose dependent. Therefore, to reduce these side effects while maintaining the therapeutic effects, reducing the drug dose or the laser irradiance of PDT is a viable option for non-CNV wet maculopathy. Currently, the most common and simple methods involve halving the PDT parameters, either the drug dosage or the laser irradiance, in clinical practice. Clinically, half-dose verteporfin PDT has been successfully used in the treatment of CSC without serious complications.^{33,34} Half-dose verteporfin in this study showed less damage to photoreceptors but did not significantly decrease the choroidal damage from PDT. However, we should note that the dose and laser parameters used in the rabbits of this study are very different compared with humans. Therefore, the study results should not be applied directly on clinical patients. Our results imply that use of the standard PDT parameters may not be necessary and that they may be too high to treat CSC. In contrast, several previous studies have reported that standard-fluence PDT was an efficacious treatment for PCV eyes, but a few studies reported that although standard-fluence PDT for PCV improved or maintained the VA at 12 months, the mean VA decreased in conjunction with an increased duration of follow-up.^{35,36} Yamashita et al. reported that reduced-fluence PDT was an effective treatment for PCV for up to 24 months. The eyes treated in their study either improved or maintained their VA, even when the baseline VA was better than 20/40. These authors concluded that choriocapillary perfusion was restored by reduced-fluence PDT and that the damage to the choroid could be prevented. They suggested that reduced-fluence PDT may be more effective than standard-fluence PDT.¹³ However, the optimal dose of verteporfin required to treat PCV or chronic CSC has not yet been established. These side effects may be prevented by selecting optimal parameters to only treat the lesion without damaging the physiological choroidal vasculature. Michels et al. used ICGA 3 months after the treatment and reported at least moderate perfusion changes in the choriocapillaris in the group that received 80% of the standard-fluence PDT (light dose, 50 J/cm²). In contrast, neither moderate nor severe changes in the choriocapillary perfusion were present at 3 months in the reduced-fluence PDT group.³⁷ In addition,

FIG. 6. This nonthermal laser itself causes damage. Note that damage to the photoreceptors was observed in the rabbits after only diode laser treatment on day 7 and day 42. (A) Laser-naive group (without laser). (B) Day 1 after laser treatment. (C) Day 7 after laser treatment (deformity of the photoreceptors was noted). (D) Day 42 after the laser treatment (photoreceptor loss was noted).

in the other study, although ICGA showed that there was mild to moderate choriocapillary nonperfusion of the irradiated area 1 week after treatment in 57% of the 28 eyes studied, choriocapillary perfusion was restored in 97% of the eyes by 3 months. These findings demonstrate that, in contrast to the standard-fluence PDT treatment outcomes, reduced-fluence PDT might prevent damage to the choroid and the development of severe hemorrhage.¹³

These clinical observations from previous studies have been corroborated in this animal study. Among the 3 PDT parameters—verteporfin dose, laser irradiance, and photosensitizing time—the laser irradiance was the most important factor influencing the collateral retinal damage after PDT treatment. Reducing the values of the PDT parameters tended to reduce the retinal damage. Clinically, half the laser irradiance induced significantly less collateral damage to the chorioretinal tissue. Therefore, in the treatment of choroidal lesions such as CSC or PCV, reducing the laser irradiance could effectively reduce the collateral damage to the retina. The results of this study are crucial for choosing optimal parameters for these diseases and preventing damage to the physiological choroid. It would have been interesting to combine the use of a half-dose of verteporfin with a half-laser irradiance. The optimal dosage of PDT that causes minimal side effects and maximizes the treatment effectiveness for each individual disease and condition needs to be further investigated.

In conclusion, among the parameters of PDT, laser irradiance was the most important factor in terms of the collateral damage caused by PDT. Half the laser irradiance induced less retinal damage in comparison to a half the drug dosage or half the photosensitizing time. However, the optimal parameter settings to balance the prevention of retinal damage with the effectiveness of the treatment must be determined.

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Author Disclosure Statement

None of the authors has any proprietary interest or financial interest related to the article.

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