Influence of Pulse Duration and Pulse Number in Selective RPE Laser Treatment

Carsten Framme, MD,[1,2]* Georg Schuele, PhD,[2] Johann Roider, MD,[1] Reginald Birngruber, PhD,[2] and Ralf Brinkmann, MSc[2]
[1]*University Eye Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93042 Regensburg, Germany*
[2]*Medical Laser Center Luebeck, Peter-Monnik-Weg 4, 23562 Luebeck, Germany*

Background and Objectives: The therapeutic effect of laser treatment for macular diseases is related to the damage to the retinal pigment epithelium (RPE) and the subsequent restoration of the defect due to RPE proliferation. In contrast to conventional laser treatment, it is possible to damage the RPE selectively and to spare the photoreceptors by using repetitive microsecond laser pulses. It was the aim of the study to investigate the influence of pulse duration and number of pulses on angiographically and ophthalmoscopically visible retinal damage thresholds in order to optimize treatment modalities.
Study Design/Materials and Methods: In total, 625 laser lesions with various parameters were applied to the retina in 11 eyes of 6 Chinchilla breed rabbits using an experimental laser system (Nd:YLF at 527 nm). Pulse duration (1.7 microseconds and 200 nanoseconds) and number of pulses (100, 10, and 1 pulses) were varied at a constant repetition rate of 100 Hz. Damage thresholds were determined in terms of ophthalmoscopic and fluorescein angiographic visibility, and the therapeutic window (TW; angiographic ED_{50} vs. ophthalmoscopic ED_{50}) as well as the safety range (SR; angiographic ED_{84} vs. ophthalmoscopic ED_{16}) between both thresholds were calculated. Selected laser lesions were evaluated by histology.
Results: Generally, the ED_{50} radiant exposure for angiographic visibility decreases for shorter laser pulses and with an increase in the number of pulses. The TW for both pulse durations (1.7 microseconds and 200 nanoseconds) was wider with 100 pulses than with single pulses. The widest TW was found for 100 pulses at 200 nanoseconds pulse duration (5.9-fold above the angiographic threshold), and the smallest TW with a factor of 1.6 was found for 1.7 microseconds single pulses. In terms of SR, only irradiation with 100 pulses at 200 nanoseconds pulse duration was associated with a ratio > 2. Independently of pulse duration, histological examination of laser sites 1 hour after irradiation revealed widely intact photoreceptors, while the underlying RPE was damaged.
Conclusions: Pulse duration and number of pulses have a significant influence on RPE damage thresholds and consecutively on TW and SR. Because fundus pigmentation in humans may vary intra- and interindividually by a factor of 2, a large TW and ideally also a large SR should be ensured in a clinical treatment context. In rabbits, the safety range with 200 nanoseconds pulses is higher than with the pulse duration of 1.7 microseconds currently in clinical use. These findings suggest the need for clinical pilot studies to prove whether these results can be transposed to the situation in humans. Lasers Surg. Med. 34:206–215, 2004. © 2004 Wiley-Liss, Inc.

Key words: RPE; laser photocoagulation; selective microphotocoagulation; threshold determination; photoreceptors; selective RPE laser treatment

INTRODUCTION

Conventional retinal laser treatment is usually performed using the continuous wave (CW) argon laser (514 nm). Generally, exposure times are 100–200 milliseconds. About 50–60% of the incident light is absorbed and converted to heat by the retinal pigment epithelium (RPE) [1]. The long irradiation time with CW laser photocoagulation leads to irreversible destruction of the outer and inner segments of the neuroretina due to protein denaturation [2–5], resulting in irreversible laser scotoma.

Many macular diseases, for example, diabetic maculopathy, drusen maculopathy, or central serous retinopathy, are thought to be associated merely with a decline in RPE cell function [6,7]. Similarly, the beneficial effect of laser treatment appears to be only RPE-related. Thus the effects of laser treatment on the fundus have been studied by several groups. It has been shown that CW argon laser photocoagulation of monkey and human fundus causes RPE necrosis and lifting of the RPE from Bruch's membrane [8], and budding of individual RPE cells [9]. Within 7 days after laser photocoagulation, intact bystander RPE cells migrate and proliferate to cover the defect, resulting in an intact RPE barrier [10] and multilayered RPE formation in the area of laser irradiation [8–14].

Similar responses have been reported in histological studies in animals following selective RPE laser treatment [15]. It has been postulated that cell proliferation enhances the pump mechanism and produces the clinical effects of reducing subretinal fluid and improving macular pathology [15–17]. However, when conventional laser photocoagulation is applied, the benefits are always accompanied by laser scotoma due to photoreceptor destruction.

Thus the selective destruction of RPE cells without causing adverse effects to the neuroretina, and to the photoreceptors in particular, might be an appropriate treatment in this context [15,16]. This selective damage of RPE cells was first demonstrated by Roider using 5 microseconds argon laser pulses at 514 nm with a repetition rate of 500 Hz [15]. By irradiating the fundus with a train of microsecond laser pulses, high peak temperatures were achieved around the melanosomes. This led to RPE cell damage, with only a low sublethal temperature increase in the adjacent tissue structures due to the short pulse duration used [18]. These selective effects were invisible on ophthalmoscopy and could be visualized only by angiography because the blood-retina barrier was damaged and the intravenously injected dye leaked through the damaged areas [19]. Selective destruction of RPE cells with sparing of photoreceptors was proven by histological examinations at different times post treatment. A first clinical trial using a neodymium-YLF (Nd:YLF) laser system with 1.7 microseconds (527 nm, 100 pulses, 500 Hz) also proved the concept of selective RPE destruction and subsequent healing by RPE migration and proliferation, leading to enhanced cellular pump function [16,19].

The aim of this work was to investigate the dependence of RPE damage thresholds on a variety of laser parameters using a Nd:YLF laser at 527 nm. The rabbit retina was used to study the influence of the number of pulses in the laser treatment from 100 pulses down to single pulses, and the influence of pulse duration (1.7 microseconds and 200 nanoseconds). In contrast to previous studies, the repetition rate in the present study was reduced from 500 Hz [20] down to 100 Hz (in trains of 10 and 100 pulses) in order to minimize heat accumulation in the neurosensory tissue. Determination of the ophthalmoscopic and angiographic damage thresholds provided information on the therapeutic window (TW) and the safety range (SR) between the two thresholds, and yielded pointers to the safety of treatment with the particular laser parameters.

MATERIALS AND METHODS

Laser

An arc-lamp excited, intracavity frequency-doubled Nd:YLF laser (Quantronix, Inc., Setauket, NY, USA, model 527DP-H) was modified with an active feedback electro-optical Q-switched system to generate variable pulse durations up to several microseconds at a wavelength of 527 nm. When the system is operated in the normal Q-switched mode, the laser typically emits 200 nanoseconds pulses. In order to extend pulse duration up to 5 microseconds, a transient high-voltage course was applied to the Pockel's

cell to increase cavity losses during pulse emission and to stretch the laser pulse [21]. The energy was transmitted by a 105 μm core diameter fiber (Ceram Optec, GmbH Bonn, Germany. Optran UV-A 105/125/250, NA = 0.1), and the length of the fiber was 50 m in order to minimize spatial intensity modulation due to speckle formation at the distal fiber tip. This fiber was directly coupled to the slit lamp fiber (Zeiss, Ø160 μm, NA 0.1).

For the experiments, pulse durations of 1.7 microseconds and 200 nanoseconds were used, each with 100 pulses, 10 pulses, and single pulses. A constant repetition rate of 100 Hz was adjusted for the experiments with 100 and 10 pulses.

Settings

The laser beam was delivered to a clinical ophthalmic laser slit lamp (SL/L 30, Zeiss, Oberkochen, Germany). The laser beam from the fiber tip was imaged onto the retinal surface of the macular area in the rabbit eye using a Goldmann contact lens with a magnification of 1.1. Calculations showed that use of a planoconcave contact lens in cycloplegic emmetropic rabbit eyes translates the laser focus of 160 μm with respect to the lens magnification into a retinal spot size of 116 μm [22]. A green helium-neon laser beam (543 nm) was used as the aiming laser.

Animals

The treatment of experimental animals in this study was in compliance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Chinchilla breed rabbits were used because the density and location of light-absorbing pigments in the fundus are relatively uniform and similar to those of the human eye [1]. The animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride 2:1, and they were placed in a special holder system, which allowed movements in all directions. The contact lens was placed onto the mydriatic eye using methylcellulose as a contact gel. The lens was locked at the animal holder to prevent unfavorable movements.

Laser Treatment and Documentation

Ophthalmoscopically visible marker lesions were used in all eyes to provide orientation for the non-visible RPE laser irradiation. Nine lesions were therefore usually placed in the macular area of about 2 mm × 2 mm. These lesions were documented graphically. The test lesions were placed between these marker lesions and again documented graphically and in tabular format (laser parameter, energy, ophthalmoscopic, and angiographic visibility). About 40–70 test lesions were placed in one eye. The first laser lesions were always applied using high energy levels producing ophthalmoscopically visible spots; stepwise energy decreases led to ophthalmoscopically invisible laser lesions. Consequently, most of the test lesions were invisible. Thirty minutes after application of the laser lesions, colored fundus photographs were taken with a fundus camera (Carl Zeiss, Oberkochen). Afterwards standard fluorescein

angiography was performed with injection of 0.5 ml 10% fluorescein sodium into the ear vein.

Two endpoints for RPE damage were chosen for evaluation: lesion visibility on ophthalmoscopy and on fluorescein angiography. The difference between the two endpoints can be clearly seen from a typical fundus photograph and the corresponding fluorescein angiogram (Fig. 1).

The dichotomous experimental data—0 for a non-visible and 1 for a visible lesion—at the different laser pulse energies were analyzed using Probit analysis [23] with the SPSS software package. The data from all eyes treated with one set of parameters were analyzed as one data set. The 50% probability threshold ED_{50} and the standard deviation width ED_{16} and ED_{84} of the logarithmic normal distribution were determined for the ophthalmoscopic and angiographic thresholds. The term "therapeutic window (TW)" has been introduced in the context of selective RPE laser treatment as the ratio of ophthalmoscopic ED_{50} and angiographic ED_{50} [20,24]. The safety range (SR) is also evaluated as the ratio of the ophthalmoscopic ED_{16} and the angiographic ED_{84}. This value also takes account of the standard deviation of the adjusted logarithmic normal distribution, or more obviously, the slope of the Probit plot [25]. Thus the "real" therapeutic window can be calculated more precisely (see also Discussion). Finally, the speckle factor of the multimode fiber used was taken into account to correct the measured data for maximum radiant exposure. It is known that interference due to transmission of laser light through multimode fibers causes intensity modulations known as speckles. This effect is based on the optical path length difference of the different fiber modes. If the path length difference is smaller than the coherence length of the laser, speckles are produced by interference. For the Nd:YLF-system with a long slit-lamp fiber, the speckle factor was $F_{Nd:YLF} = 1.225$ (no speckle: $F = 1$), indicating almost regular and homogenous speckle formation [20]. This factor is included in all radiant exposure values cited in this study.

Histology

Selected laser lesions were evaluated histologically 1 hour after treatment. Attention was focused on the laser parameter sets 1.7 microseconds / 10 pulses and 200 nanoseconds/10 pulses. For each of the parameters examined, lesions applied with 1.2-fold angiographic ED_{50} and 0.8-fold ophthalmoscopic ED_{50} were considered. Thus, as also expected clinically, this ensures RPE damage (1.2-fold ED_{50}^{ang}), which is ophthalmoscopically not visible and suggests intact neuroretina (0.8-fold ED_{50}^{oph}).

The eyes were enucleated under deep anesthesia in vivo to avoid choroidal swelling. The animal was sacrificed immediately after enucleation. The globes were then incised anterior to the equator, and immersed with 2.5% Karnofsky's solution. The complete globe was then placed into this solution. Thirty minutes later, the posterior optic cup was cut from the anterior segment and the posterior globe was replaced in the fixative solution. After a further 30 minutes, the samples were fixed in 2.5% glutaraldehyde and postfixed in Dalto's osmium fixative, dehydrated in alcohol and embedded in expoxy resin (Epon). Ultra-thin
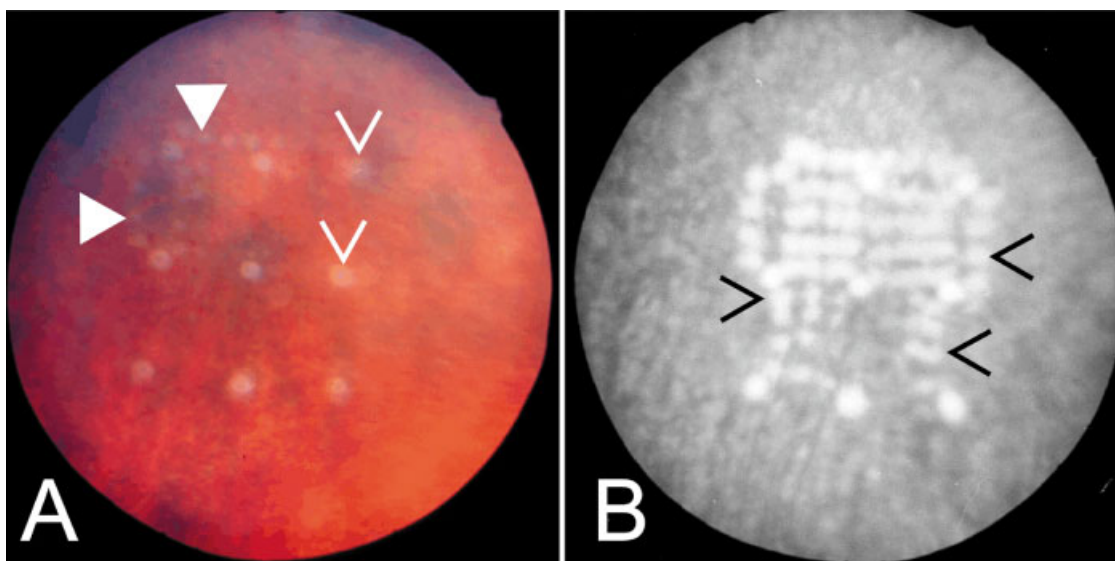

Fig. 1. **A**: Fundus photograph obtained 30 minutes after irradiation with repetitive 1.7 microseconds and 200 nanoseconds laser pulses (10 pulses, 100 Hz). The white open wedges aim to the large orientation markers. The white triangles mark some of the visible lesions, which were non-visible immediately after irradiation. **B**: Corresponding fluorescin angiography of the fundus obtained 30 minutes after exposure. All ophthalmoscopic visible spots from Figure 1A are also angiographic visible. But in addition, the ophthalmoscopic non-visible RPE damage can be imaged by angiographically leakage, due to the diffusion of choroidal fluorescein through the broken blood-retina barrier. Some of the only angiographic visible spots are marked by black open wedges. [Figure can be viewed in color online via www.interscience.wiley.com.]

sections were stained with uranyl acetate. One-micrometer serial sections were cut until the center of the lesion was reached.

RESULTS

Laser experiments were performed in 11 eyes from six animals. In total, 625 lesions were evaluated (1.7 microseconds: n = 259; 200 nanoseconds: n = 366). The ED_{16}, ED_{50}, and ED_{84} radiant exposures determined for all parameters (ophthalmoscopic and angiographic visibility) are summarized in Table 1.

Most of the laser lesions applied to the retina were ophthalmoscopically non-visible. Macroscopic disruptive effects, such as bleeding, never occurred at therapeutic energy levels. At high energy levels, blanching of the retina occurred comparable to the whitening seen after conventional laser photocoagulation. This was a continuous, not an abrupt process. In terms of the parameters used in this study, the necessary energy was between 1.6 and 5.9 times higher than the angiographic threshold (Table 1). Bleeding never occurred at these energy levels. With increasing pulse energies (3–4 times higher than the ophthalmoscopic ED_{50}), long-lasting bubble formation was noted in the center of the laser spot, and this has already been described [24]. Hemorrhage was then seen at high energy levels (about 5–6 times higher than the ophthalmoscopic ED_{50}), which is nearly twice as high as the threshold for macroscopically visible, long-lasting bubble formation. These hemorrhages were noted in some marker lesions. Hemorrhage never occurred without this long-lasting bubble formation.

It was generally found that the angiographic damage threshold for radiant exposure decreased with shorter pulse duration and also with increasing number of pulses applied to the retina. Thus the lowest threshold was found for the parameter set 200 nanoseconds pulse duration with 100 pulses, whereas the highest threshold was found for 1.7 microseconds duration with single pulses. Even with single pulses, no hemorrhage and retinal disruption were seen at energy levels within the therapeutic range (Table 1). The ophthalmoscopic thresholds generally did not follow the trend described for the angiographic thresholds; however, the percentage threshold changes were smaller than those for angiography. Thus the dependence on pulse duration and number of pulses was found to be inconsistent.

When pulse energy was plotted against number of pulses, it was found that the angiographic thresholds—more for 200 nanoseconds pulses than for 1.7 microseconds pulses—decreased with the number of pulses, which is in accordance with the empirical $n^{-1/4}$ law [$ED_{50}(n) = n^{-1/4}*ED_{50}(n = 1)$]. In this plot, the $ED_{50}(n = 1)$ value correlated strongly with the measured value. By contrast, a good correlation was not found for the ophthalmoscopic thresholds (Fig. 2).

Since angiographic damage thresholds decrease with the number of pulses more markedly than ophthalmoscopic thresholds, the TW became wider with number of pulses for both pulse durations: the largest TW (factor: 5.9) was found for the parameter set 200 nanoseconds/100 pulses and the smallest TW (factor: 1.6) for the set 1.7 microseconds/single pulse. In terms of SR, the only parameter set with a therapeutic difference factor > 2 was irradiation with 200 nanoseconds pulse duration/100 pulses followed by 1.7 microseconds pulse duration/100 pulses with an SR of 1.7 (Table 1, Fig. 3). Thus the therapeutic width was always greater for 200 nanoseconds pulse duration than for 1.7 microseconds pulse duration.

Histological assessment of 200 nanoseconds laser lesions (10 pulses) applied with 23 μJ (266 mJ/cm^2 = 0.8 times ED_{50}^{oph}) revealed damage chiefly to the RPE and partially to the outer photoreceptor segments (Fig. 4). The RPE was completely destroyed and condensed, and debris was detected on Bruch's membrane, which itself was intact. The choriocapillaris showed open vessels. The outer photoreceptor segments appeared relaxed but mainly intact (Fig. 5). Staining of the outer segments was weaker at the lesion site, and more enhanced for 0.8-fold ED_{50}^{oph} than for lower energy levels. The inner photoreceptor segments as well as the other neurosensory layers were intact (Table 2). At 11 μJ (127 mJ/cm^2 = 1.2 times ED_{50}^{ang}), histological assessment revealed similar findings with less relaxation of the outer segments (Fig. 6). By comparison, histological assessment of ophthalmoscopically visible laser lesions applied with 200 nanoseconds/100 pulses at 30 μJ (346 mJ/cm^2 = 1.4-fold ED_{50}^{oph}) showed intact choriocapillaris and Bruch's membrane, completely damaged RPE and outer segments but intact inner segments with vacuoles (Fig. 7). Thus even the lesion is ophthalmoscopically visible, suggesting neurosensory damage, and the extent of denaturation is maximally limited compared with conventional laser

TABLE 1. Summary of All Speckle-Corrected ED_{16}, ED_{50}, and ED_{84} Radiant Exposures, the Therapeutic Window (TW) as the Ratio of the Ophthalmoscopic to Angiographic Thresholds, and the Safety Range (SR) as the Ratio of the Ophthalmoscopic ED_{16} and Angiographic ED_{84}

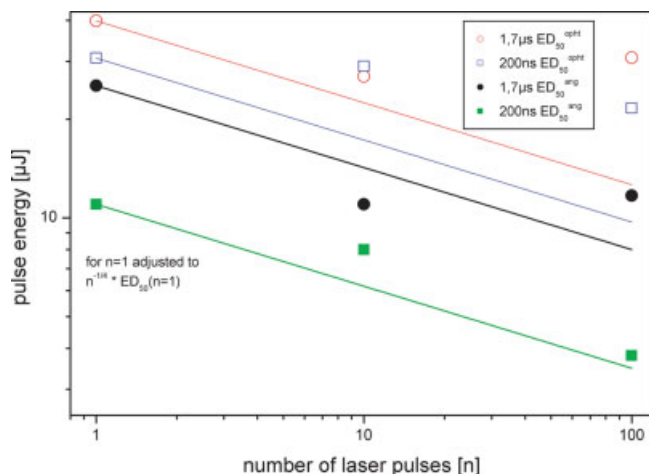| Parameter	ED_{16}^{oph} (mJ/cm^2)	ED_{50}^{oph} (mJ/cm^2)	ED_{50}^{ang} (mJ/cm^2)	ED_{84}^{ang} (mJ/cm^2)	TW ED_{50}^{oph} vs. ED_{50}^{ang}	SR ED_{16}^{oph} vs. ED_{84}^{ang}
200 nanoseconds 1 p	194	354	123	181	2.9	1.1
200 nanoseconds 10 p	223	335	89	137	3.7	1.6
200 nanoseconds 100 p	205	249	42	71	5.9	2.9
1.7 microseconds 1 p	264	461	282	448	1.6	0.6
1.7 microseconds 10 p	224	312	123	214	2.5	1.0
1.7 microseconds 100 p	263	356	131	159	2.7	1.7

Fig. 2. Pulse energy plotted versus the number of laser pulses (1, 10, and 100 pulses) for the angiographic and ophthalmoscopic ED_{50}-values (1.7 microseconds and 200 nanoseconds pulse duration). For both pulse duration, the angiographic values fit reasonable the empiric $n^{-1/4}$ law [$ED_{50}(n) = n^{-1/4}*ED_{50}(n=1)$], whereas ophthalmoscopic measurements do not as good. In this plot, the $ED_{50}(n=1)$ value is fixed to the measured value. [Figure can be viewed in color online via www.interscience.wiley.com.]

lesions. Laser lesions applied with 1.7 microseconds/10 pulses at 0.8-fold $ED_{50}{}^{oph}$ (22 μJ = 254 mJ/cm^2) were comparable with the 200 nanoseconds lesions in terms of extent of damage (Fig. 8). Thus, according to Table 2, similar damage is obtained for both pulse durations.
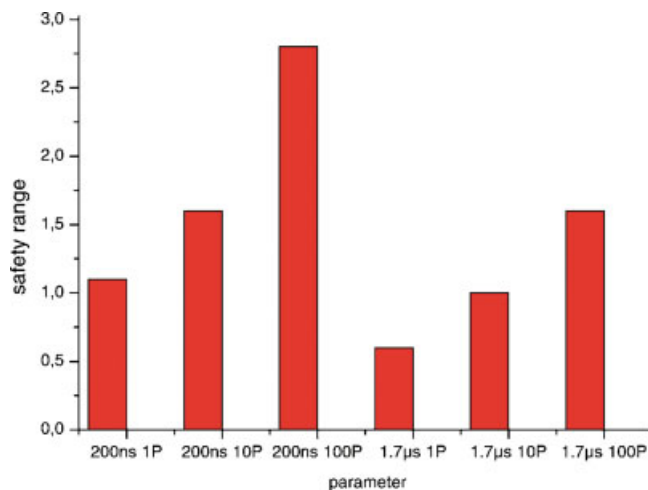

Fig. 3. Safety range (ophthalmoscopic ED_{16} vs. angiographic ED_{84}) displayed for all laser parameters tested. Irradiation using the 200 nanoseconds laser pulses shows a wider safety range than 1.7 microseconds laser pulses. [Figure can be viewed in color online via www.interscience.wiley.com.]

DISCUSSION

The aim of this study was to evaluate the influence of pulse duration and number of repetitive pulses applied to the retina on the ophthalmoscopic and angiographic damage thresholds and to determine the therapeutic window and safety range between the two thresholds. Current clinical pilot studies in treating macular diseases with this new method use repetitive 1.7 microseconds-laser pulses [16,19]. Previous calculations and early rabbit studies [15,18] have shown that these microsecond pulses are short enough to avoid destruction of the neurosensory layer: because the pulses are shorter than the thermal relaxation time of the RPE, there is no temperature increase in this tissue. Repetitive application of laser energy has always been performed to reduce the energy of each single pulse and to exploit the additive effect of a laser pulse train. This approach was thought to be important because the selective RPE damage was primarily postulated as a thermal defect due to repetitively high temperatures generated by this train of single pulses [18] and, according to Arrhenius' model, the rate of thermal damage to tissue increases markedly with temperature [26]. However, recent investigations have indicated that, in all probability, thermomechanical damage to the cells should be taken into account [27,31]. This raised the question as to whether selective laser treatment might also be possible using shorter pulses, or fewer pulses down to single pulses, without adversely affecting the neurosensory retina, for example, due to hemorrhage and disruption.

Angiographic thresholds generally fall with shorter pulse duration, as seen in this study and confirmed by the results of previous studies [20]. With shorter pulses, less heat dissipates from the melanosomes into the tissue and thus less energy is required to heat the melanosomes, followed by microbubble formation and consequent rupture of the single RPE cell [27]. Thus, the lowest ED_{50}-radiant exposures were obtained for irradiation with 200 nanoseconds pulses (100 pulses) whereas the highest values were recorded for longer single 1.7 microseconds pulses. The differences between these thresholds were great than a factor of 2—a finding that is also comparable with earlier experiments (performed with two different lasers), which achieved at least factor 2 changes between 200 nanoseconds and 5 microseconds pulse durations [20].

Ophthalmoscopic thresholds did not show this clear dependence on pulse duration. In particular, the parameter 1.7 microseconds with 10 pulses did not fit the general pattern of ophthalmoscopic and angiographic damage thresholds. This finding could not be addressed specifically because all parameters were mixed and distributed over several eyes. However, the number of eyes was relatively small, and the difference might be due to coincidence in terms of different fundus pigmentation patterns. Changes as a function of pulse duration and number of pulses were merely 30−40%, but this was in contrast to previous experiments, which showed changes of just 10−15% [20]. However, the previous study was conducted using a higher repetition rate of 500 Hz. Due to the lower repetition rate of

Fig. 4. Histology of two laser lesions applied with 200 nanoseconds, 10 pulses, 0.8-fold $ED_{50}^{oph} = 266$ mJ/cm^2 = 23 µJ (magnification 157×). Arrows mark the area of RPE laser damage. [Figure can be viewed in color online via www.interscience.wiley.com.]

100 Hz in this study, it is possible that thermal side effects caused by heat accumulation from multiple pulses were less pronounced. In fact, by reducing the repetition rate towards 100 Hz, the maximal temperature increase in retinal tissue becomes smaller [31]. Thus, thermal damage (as seen ophthalmoscopically by scattered tissue changes due to denaturation) appears to be more independent of pulse duration at high repetition rates than at lower repetition rates.

Fig. 5. Histology of a laser lesion applied with 200 nanoseconds, 10 pulses, 0.8-fold $ED_{50}^{oph} = 266$ mJ/cm^2 = 23 µJ (magnification 1,000×). The figure displays the transition from lesion (**left**) to normal RPE (**right**). The black arrow marks the border of the lesion, and the white arrow marks a condensed core of the damaged RPE cell. The outer photoreceptor segments are relaxed. [Figure can be viewed in color online via www. interscience.wiley.com.]

TABLE 2. Summary of Histological Findings for Selected Laser Parameters

Parameter	Figure 6	Figures 4 and 5	Figure 7	Figure 8
Pulse duration	200 nanoseconds	200 nanoseconds	200 nanoseconds	1.7 microseconds
Number of pulses	10	10	100	10
Pulse energy	11 μJ	23 μJ	30 μJ	22 μJ
Angiographic visibility	+	+	+	+
Ophthalmoscopic visibility	−	−	+	−
Bruch's membrane	Intact	Intact	Intact	Intact
RPE	Completely damaged; condensed cores, enhanced staining; debris on Bruch's membrane	Completely damaged; condensed cores, enhanced staining; debris on Bruch's membrane	Completely damaged; condensed cores, enhanced staining; debris on Bruch's membrane	Completely damaged; condensed cores, enhanced staining; debris on Bruch's membrane
Photoreceptors: outer segments	Weaker staining; distal ends relaxed	Weaker staining; distal ends relaxed	Relaxed; vacuoles	Weaker staining; distal ends relaxed
Photoreceptors: inner segments	Unchanged	Unchanged	Some vacuoles	Unchanged
Other retinal layers	Unchanged	Unchanged	Unchanged	Unchanged

When pulse energy was plotted against the number of pulses, it was found that the angiographic thresholds changed in accordance with the empirical $n^{-1/4}$ law $[ED_{50}(n) = n^{-1/4}*ED_{50}(n = 1)]$. This law describes the empirical fact that thresholds fall in line with the number of pulses applied to the tissue. In the literature, this law has been reported only for ophthalmoscopically visible damage [28,29]. These authors have described complete thermal damage with spot diameters of about 20 μm. With larger spot diameters, as in our experiments, ophthalmoscopically visible damage did not show any dependence [30]. This is comparable to our findings regarding ophthalmoscopic thresholds. However, angiographic thresholds—in contrast to ophthalmoscopic thresholds—appear to obey this law, especially for 200 nanoseconds pulse durations. The reasons for this remain unclear although they may be related to the larger spot sizes used in our study or to the different damage mechanism in selective RPE laser
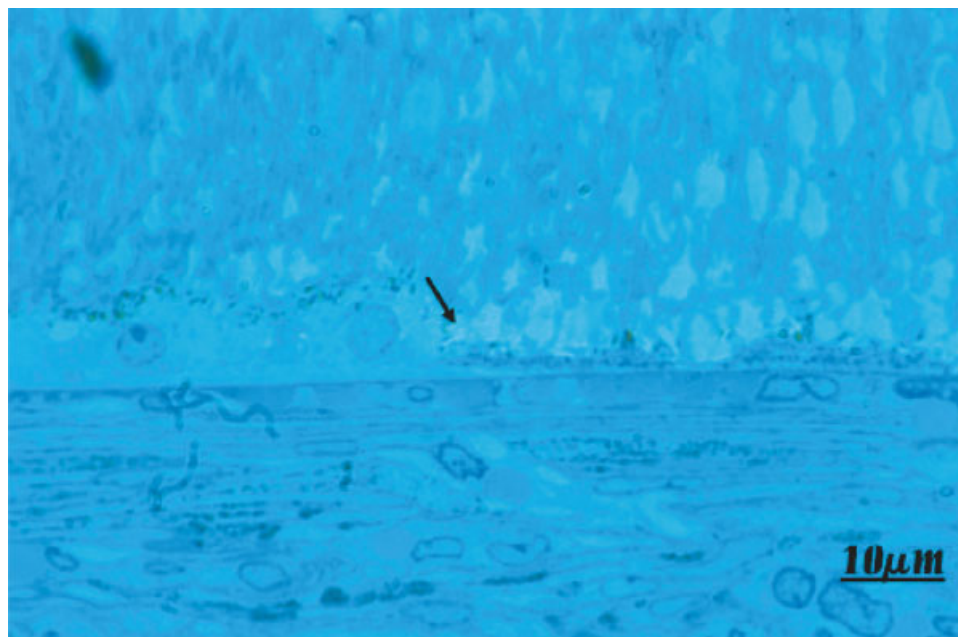

Fig. 6. Histology of a laser lesion applied with 200 nanoseconds, 10 pulses, 1.2-fold $ED_{50}^{ang} = 127$ mJ/cm^2 = 11 μJ (magnification 1,000×). The figure displays the transition from normal RPE (**left**) to laser lesion (**right**). The arrow marks the border of the lesion. [Figure can be viewed in color online via www.interscience.wiley.com.]
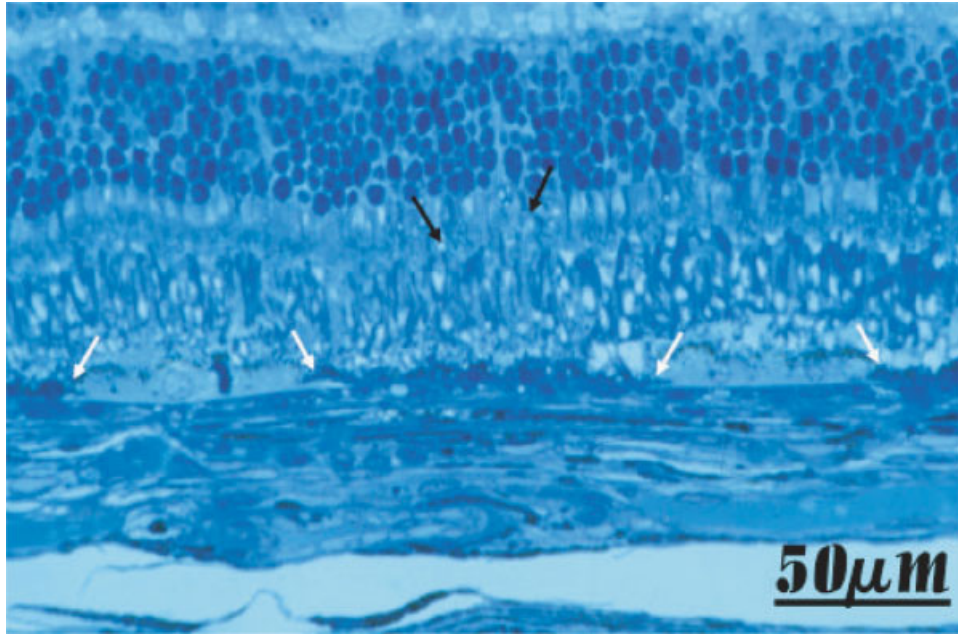
Fig. 7. Histology of an ophthalmoscopically visible laser lesion applied with 200 nanoseconds, 100 pulses, 30 μJ = 346 mJ/cm^2 (magnification 400×). The white arrows mark the borders of the lesion, the black arrow marks vacuoles in the inner photoreceptor segments. [Figure can be viewed in color online via www.interscience.wiley.com.]

treatment. On the other hand, these results may also suggest that this empirical law might be inconsistent.

Since it is known that intra- and interindividual fundus pigmentation may vary by a factor of 2 [1,32], it appears reasonable to ensure a wide therapeutic window for selective treatment. ED$_{50}$-damage thresholds are usually calculated to determine these factors [24,25]. However, according to Sliney et al., a major and recurring discussion
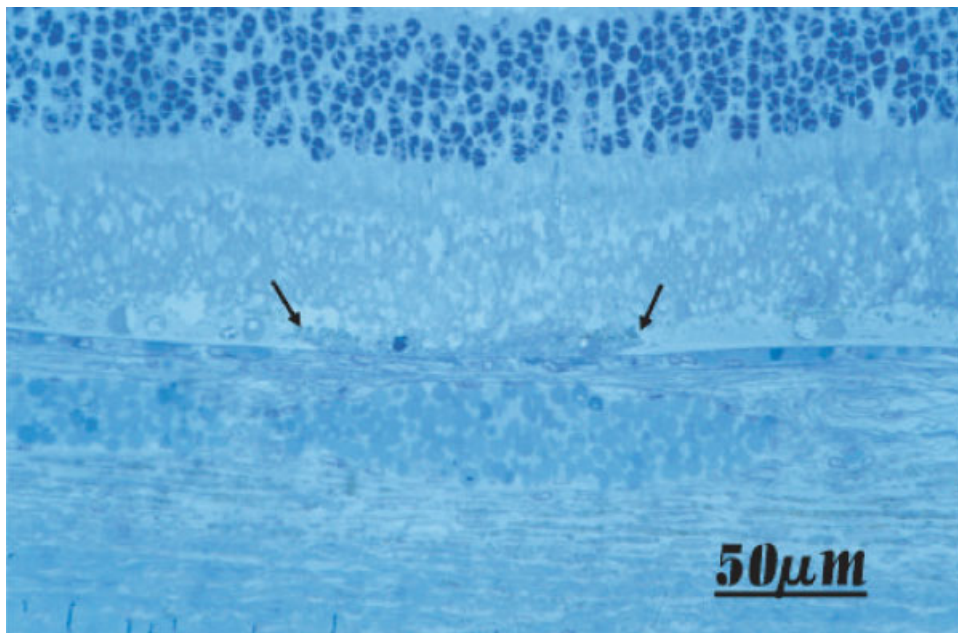

Fig. 8. Histology of a laser lesion applied with 1.7 microseconds, 10 pulses, 0.8-fold ED$_{50}$^{oph} = 254 mJ/cm^2 = 22 μJ (magnification 400×). Arrows mark the laser lesion. [Figure can be viewed in color online via www.interscience.wiley.com.]

point in laser injury studies surrounds the level of uncertainty over the threshold of injury, which relates to the slope of the transformed dose-response curve, or the "Probit plot" [25]. They further state that the most frequently cited point on the probit plot is the exposure that represents a 50% probability of injury. This value is frequently referred to as the "threshold", even though many "sub-threshold" experimental damage points exist [25]. Thus, a safety interval should be taken into account, resulting in smaller therapeutic ranges throughout our study, as indicated by the ophthalmoscopic ED_{16} and angiographic ED_{84} SR values. On this basis, the 100 pulses of 200 nanoseconds seem to be the best parameter set in this study for performing selective laser treatment. The 200 nanoseconds parameter may be superior to the 1.7 microseconds pulses because adjacent heat flow during irradiation into the neurosensory tissue is lower. However, in terms of the number of pulses, treatment with 100 pulses at the 100 Hz repetition rate has no clinical relevance since irradiation would last for 1 second, and this might interfere with eye movements. With single pulses, the safety range appears to be too small for proper selective laser treatment because the variability of fundus pigmentation must be considered. Thus, for histological examination, attention was focused on irradiation with 10 pulses, as shown in Table 2. It was demonstrated that selective damage of RPE without significant disturbance of photoreceptors is possible using either 1.7 microseconds or 200 nanoseconds at 10 pulses. Some disturbance of the outer photoreceptor segments may appear, especially if higher energy levels near the ophthalmoscopic threshold are used. However, if the inner segments and especially the cores are undamaged—as is the case—proper function should be guaranteed. Proper photoreceptor function after treatment has also been clearly demonstrated by microperimetry findings using different laser parameter sets in clinical pilot trials [19]. Because rabbit RPE is thought to resemble human RPE in terms of absorption characteristics, granular absorption structure of the melanosomes [1], and photoreceptors which are in immediate proximity to the RPE, it might be possible to extrapolate the data from this study to the situation in humans. However, it must be remembered that all the data were obtained in a small number of rabbit eyes, which showed fourfold lower angiographic damage thresholds compared with human eyes using pulse trains with a single pulse duration of 1.7 microseconds. The transposability of the therapeutic safety range to the human eye with shorter pulse durations (e.g., 200 nanoseconds) or a small number of pulses requires extremely careful investigation in clinical pilot studies. On the basis of these results in rabbit eyes, treatment with 200 nanoseconds pulses might be preferable to the 1.7 microseconds pulses due the wider therapeutic range. However, the dynamics of microbubble formation around the melanin granules should also be investigated for the shorter pulses to avoid unintentional effects, which might not be visible in histological specimens.

It is not yet clear how important are the vacuoles between the inner photoreceptor segments seen in ophthalmoscopically visible laser lesions following irradiation with 200 nanoseconds pulses at energy levels slightly above the ophthalmoscopic threshold. Because the photoreceptors themselves do not show complete damage but only enhanced structural relaxation, this may be less an indication of more extensive damage by denaturation and more an explanation for enhanced ophthalmoscopic visibility due to light scattering. To avoid such supra-threshold irradiation, an online control system has been devised, based on opto-acoustic measurements of microbubble formation around the melanosomes, which is indicative of RPE damage [31]. This makes it possible to use energy levels slightly above the angiographic damage threshold, while preventing unnecessary heat and collateral tissue damage.

CONCLUSION

Laser pulse duration and number of applied pulses are the key parameters for evaluating retinal damage thresholds. For therapeutic ophthalmic laser treatments, in particular, it is important to have a broad overview of the possible parameter range. In this study, we have demonstrated in rabbits that the gap between angiographically and ophthalmoscopically visible retinal damage depends on these two parameters (laser pulse duration and number of applied pulses), and this finding is clinically important for the newly developed technique of selective RPE treatment.

Lower damage thresholds were associated both with short laser pulses and with high numbers of applied pulses. Both the therapeutic window and the safety range are increased for the 200 nanoseconds pulses compared with the 1.7 microseconds laser pulses, which are currently in clinical use for patient treatment. These two laser pulse durations showed similar histological damage to the RPE, with minimal side effects to photoreceptors. The parameter set with the highest number of applied laser pulses consistently showed the best therapeutic window and safety range. On the basis of these data, selective RPE treatment with a high number of 200 nanoseconds laser pulses might be preferable to the currently used 1.7 microseconds laser pulses.

REFERENCES

1. Gabel VP, Birngruber R, Hillenkamp F. Visible and near infrared light absorption in pigment epithelium and choroid. In: Shimizu K, editor. International Congress Series No. 450, XXIII Concilium Ophthalmologicum, Kyoto. Princeton, NJ: Excerpta Medica; 1978. pp 658−662.
2. Birngruber R. Thermal modeling in biological tissue. New York: Plenum Press, 1980.
3. Lorenz B, Birngruber R, Vogel A. Quantifizierung der Wellenlängenabhängigkeit laserinduzierter Aderhauteffekte. Fortschr Ophthalmol 1989;86:644−654.
4. Marshall J, Mellerio J. Pathological development of retinal laser photocoagulations. Exp Eye Res 1968;7:225−230.
5. Wallow IH, Birngruber R, Gabel VP, Hillenkamp F, Lund OE. Netzhautreaktion nach Intensivlichtbestrahlung. Adv Ophthalmol 1975;31:159−232.
6. Bresnick G. Diabetic maculopathy: A critical review highlighting diffuse macular edema. Ophthalmology 1983;90:1301−1317.

7. Spitznas M. Pathogenesis of central serous retinopathy: A new working hypothesis. Graefes Arch Clin Exp Ophthalmology 1986;224:321–324.
8. Smiddy WE, Fine SL, Quigley HA, Dunkelberger G, Hohmann RM, Addicks EM. Cell proliferation after laser photocoagulation in primate retina: An autoradiographic study. Arch Ophthalmol 1986;104:1065–1069.
9. Marshall J, Clover G, Rothery S. Some new findings on retinal irradiation by krypton and argon lasers. Doc Ophthalmol 1984;36:21–37.
10. Del Priore LV, Glaser BM, Quigley HA, Green R. Response of pig retinal pigment epithelium to laser photocoagulation in organ culture. Arch Ophthalmol 1989;107:119–122.
11. Framme C, Kobuch K, Eckert E, Monzer J, Roider J. RPE in the perfusion culture and its response to laser application—Preliminary report. Ophthalmologica 2002;216(5):320–328.
12. Matsumoto M, Yoshimura N, Honda Y. Increased production of transforming growth factor-b2 from cultures human retinal pigment epithelial cells by photocoagulation. Invest Ophthalmol Vis Sci 1994;35:4245–4252.
13. Pollack A, Heriot WJ, Henkind P. Cellular processes causing defects in Bruch's membrane following krypton laser photocoagulation. Ophthalmology 1986;93:1113–1119.
14. Wallow IH. Repair of the pigment epithelial barrier following photocoagulation. Arch Ophthalmol 1984;102:126–135.
15. Roider J, Michaud NA, Flotte TJ, Birngruber R. Response of the retinal pigment epithelium to selective photocoagulation. Arch Ophthalmol 1992;110:1786–1792.
16. Roider J, Brinkmann R, Wirbelauer C, Laqua H, Birngruber R. Subthreshold (retinal pigment epithelium) photocoagulation in macular diseases: A pilot study. Br J Ophthalmol 2000;84:40–47.
17. Framme C, Brinkmann R, Birngruber R, Roider J. Autofluorescence imaging after selective RPE laser treatment in macular diseases and clinical outcome: A pilot study. Br J Ophthalmol 2002;86(10):1099–1106.
18. Roider J, Hillenkamp F, Flotte TJ, Birngruber R. Microphotocoagulation: Selective effects of repetitive short laser pulses. Proc Nat Acad Sci USA 1993;90:8643–8647.
19. Roider J, Wirbelauer C, Brinkmann R, Laqua H, Birngruber R. Control and detection of subthreshold effects in the first clinical trial of macular diseases. Invest Ophthalmol Vis Sci 1998;39:104.
20. Framme C, Schüle G, Roider J, Kracht D, Birngruber R, Brinkmann R. Threshold determinations for selective RPE damage with repetitive pulsed microsecond laser systems in rabbits. Ophthalmic Surg Lasers 2002;33(5):400–409.
21. Brinkmann R, Meyer W, Engelhardt R, Walling JC. Laser induced shockwave lithotripsy by use of an 1 µs Alexandrite laser. Proc SPIE 1990;1200:67–74.
22. Birngruber R. Die Lichtbelastung unbehandelter Netzhautareale bei der Photokoagulation. Fortschr Ophthalmol 1984; 81:147–149.
23. Finney DJ. Probit analysis, 3rd edition. London: Cambridge University Press, 1971.
24. Roider J, Lindemann C, El-Hifnawi ES, Laqua H, Birngruber R. Therapeutic range of repetitive laser exposures in selective RPE photocoagulation. Graefe's Arch Clin Exp Ophthalmol 1998;236:213–219.
25. Sliney DH, Mellerio J, Gabel VP, Schulmeister K. What is the meaning of threshold in laser injury experiments? Implications for human exposure limits. Health Phys 2002;82(3): 335–347.
26. Birngruber R, Gabel VP, Hillenkamp F. Experimental studies of laser thermal retinal injury. Health Phys 1983; 44:519–531.
27. Brinkmann R, Hüttmann G, Rögener J, Roider J, Birngruber R, Lin CP. Origin of retinal pigment epithelium cell damage by pulsed laser irradiance in the nanosecond to microsecond time regimen. Lasers Surg Med 2000;27:451–464.
28. Ham W, Mueller H, Wolbarsht ML, Sliney DH. Evaluation of retinal exposures from repetitively pulsed and scanning lasers. Health Phys 1988;54:337–344.
29. Sliney DH, Marshall J. Tissue specific damage to the retinal pigment epithelium: Mechanisms and therapeutic implication. Lasers and Light in Ophthalmology 1992;5:17–28.
30. Greiss GA, Blankenstein MF, Williford GG. Ocular damage from multiple-pulse laser exposure. Health Phys 1980;39: 921–927.
31. Schüle G, Joachimmeyer E, Framme C, Roider J, Birngruber R, Brinkmann R. Optoacoustic detection of selective RPE cell damage during µs-laser irradiation. Proc SPIE 2001; Laser-Tissue Interactions, Therapeutic Applications, and Photodynamic Therapy, Vol. 4433, pp. 92–96.
32. Hammond BR, Caruso-Avery M. Macular pigment optical density in a southwestern sample. IOVS 2000;41:1492–1497.