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SPIE.

Event: European Conferences on Biomedical Optics, 2019, Munich, Germany

source: <https://doi.org/10.24451/arbora.9194> | downloaded: 5.3.2020

***Ex-vivo* investigation of different μ s laser pulse durations for selective retina therapy**

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ABSTRACT

Selective retina therapy (SRT) is currently used in clinical studies to treat several chorioretinal diseases. For SRT a laser pulse duration of 1.7 μ s is currently used. At this pulse duration the retinal pigment epithelium (RPE) cells are destroyed by transient microbubbles without damaging the neuronal retina. So far it is unclear whether slightly longer laser pulses are still acting thermomechanically or whether thermal effects show responsible for cell damage close above damage threshold. In order to investigate the damage threshold increase with pulse duration, a novel laser with adjustable pulse duration in the range of 2-20 μ s was used to investigate RPE damage on *ex-vivo* porcine RPE explants. The specimen were fixed in an eye model and were exposed to laser pulse energies ranging from 15-150 μ J with a top hat square of 120x120 μ m², exhibiting a spatial intensity modulation factor of 1,3. Viability tests using binary evaluation result in threshold values with peak radiant exposures of 233 mJ/cm² and 389 mJ/cm² for 2 μ s and 20 μ s laser durations, respectively. An almost logarithmic increase of the threshold radiant exposure over pulse duration was found.

Keywords: selective retina therapy, SRT, retinal pigment epithelium, eye model, ED-values, intensity modulation factor, IMF

1. INTRODUCTION

In the last decade, the development of retinal laser therapy for chorioretinal diseases has become more and more important. To avoid the damage of the neuronal retina the selective retina therapy (SRT) was evaluated ^[1]. SRT intended a laser treatment of a less functional retinal pigment epithelium (RPE), which is associated with the pathogenesis of chorioretinal diseases such as diabetic macular edema ^[2] and central serous retinopathy ^[3]. By using repetitive microsecond pulses around the thermal confinement time, SRT induces local microbubbles at the RPE-melanosomes and disintegrate the RPE by a thermomechanical effect without affecting the surrounding tissue ^[4, 5]. The clinical use and benefit of SRT has been already shown in clinical applications for diabetic macular edema and central serous retinopathy ^[2, 6-8]. However, when the pulse durations are longer than 50 μ s non-selective thermal effects govern the damage ^{[9], [10]}. Furthermore, it should not be neglected that the ratio of peak intensity and average intensity across the area of the laser beam (intensity modulation factor (IMF)) has an important influence on the selectivity of the retina laser therapy as well as the general clinical safety of the laser. The modulations of the intensity in the target area depend on the laser medium and resonator, geometry of the transmission fibre (size, round, angular), the length of transmission fibre and the mechanical winding of the transmission fibre. With increasing IMF, the clinical safety window becomes smaller and unwanted effects of laser irradiation may occur before the desired RPE damage is achieved over the entire irradiated area. As investigation target RPE-choroid-sclera explants turned out to allow an accurate experimental environment for laser-tissue interaction. Nevertheless, the duration of the measurement is limited by the rapid reduction of tissue viability ^[11].

In this study we investigated the damage thresholds on RPE in an *ex-vivo* eye model after laser irradiation with a new high power laser system in the green spectral range which provides pulse durations adjustable in the range of 2-20 μ s.

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2. MATERIAL AND METHODS

2.1 Laser system

An experimental SRT laser (MERILAS, Meridian, Thun, CH) emitting a wavelength of 532 nm was used. Radiation was transmitted via a multimode square core (50x50 μm^2) fibre (NA = 0,1). The laser was integrated in an opto-mechanically upgraded diagnostic imaging platform (SPECTRALIS HRA+OCT, Heidelberg Engineering, Heidelberg, DE). The resulting, so called Spectralis Centaurus system (HuCE-optoLab, Bern University of Applied Sciences, Biel, CH) combines SRT with optical coherence tomography (OCT). The Spectralis Centaurus system is for ophthalmological use and enables variable application patterns (10x10 lesions in this case) and a squared spot of 120x120 μm . In this study the RPE-explants were treated with single laser pulse durations of 2-20 μs and laser pulse energy range of 15-150 μJ by raster scanning the laser across the RPE-explant. Furthermore, photocoagulation marker lesions were set for orientation with 200 ms pulse duration and a power of 200 mW. Every laser parameter pair appeared twice in the pattern and each marker and lesion were manually triggered. An IR-tracking camera served as live image for the treatment.

2.2 Intensity modulation factor (IMF) analysis

In order to compare lateral intensity modulations on the target tissue, the IMF is calculated according to

$$IMF = \frac{\text{peak radiant exposure}}{\text{average radiant exposure across the area}} \quad (1)$$

An ideal top hat profile therefore has an IMF=1. To calculate the IMF, the fibre end of the experimental SRT laser was imaged with a magnification of 9.81 on a beam analyser camera (Thorlabs BC 106 VIS, Dachau, Germany). Different laser parameters were set and investigated between 2 μs at 20 μJ to 20 μs at 420 μJ .

The IMF is calculated from a Matlab routine implemented at the Medical Laser Center Lübeck. Therefore the expected spot diameter, the pixel size of the beam camera and used magnification must be specified. From the given parameters, a circle is searched in the recorded image data of the beam analyser camera. This is done using the canny edge detection, which identifies the edges of the laser profile. Then the noise outside the circle is defined by a threshold determination. The average value of the noise is calculated and subtracted from all pixel values. Subsequently, an ellipse is searched in the image, which represents the beam and fits to the previously defined circle. A mask is created in which the IMF is determined. In addition to this beam profile parameter, the maximum grey value, the minimum and maximum diameter, the roundness of the spot and the steepness of the edge can be calculated.

2.3 RPE preparation and artificial eye

As experimental model RPE-choroid-sclera-explants from enucleated porcine eyes were used. For the preparation the anterior parts of the eye, lens and vitreous body were removed (Fig. 1 a-b) according to Klettner and Miura^[12]. A highly pigmented RPE-choroid-sclera-explant was maintained in a Dulbecco's Modified Eagle Medium (DMEM, high glucose, Merck KGaA, Darmstadt, Germany) mixed with 10 % porcine serum, 1 mM sodium pyruvate, and antibiotic antimicrobial agents^[12] on a heating plate at 37°C (Fig. 1c). For the SRT-treatment, the RPE was retained in a customised eye model of the Medical Laser Center Lübeck. The eye model consists of a cuvette, a moveable explant holder and an integrated hard lens at the front and is located in front of the laser system. For the preservation of the viability of the tissue, the cuvette is filled with Dulbecco's phosphate buffered saline without calcium chloride (CaCl₂) und magnesium chloride (MgCl₂) (PBS (-)) (Merck KGaA, Darmstadt, Germany).

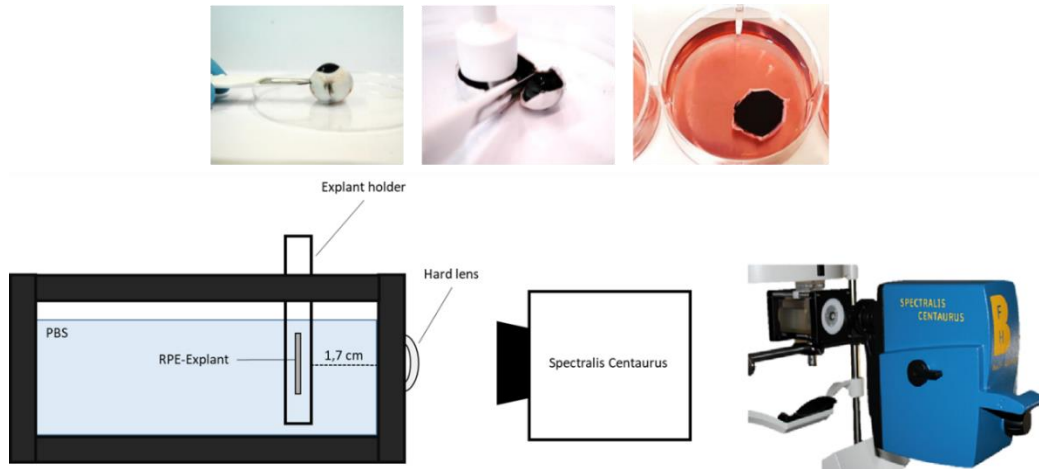


Figure 1: On top preparation steps for porcine RPE-choroid-sclera-explants. After removing lens and vitreous body, the explants are maintained in a culture medium on a heating plate. At the bottom the eye model is shown schematically. It consists of a cuvette with an integrated hard lens. The RPE-choroid-sclera-explant is placed in an explant holder 1,7 cm to the inner edge of the cuvette. The eye model is filled with PBS(-) and is located in front of the laser system.

2.4 Calcein-AM assay and binary evaluation of the RPE-damage

The viability of the RPE-cells was tested by a calcein-AM-assay directly after SRT-irradiation. Calcein-AM diffused into the cell and conformed to calcein which showed a green fluorescence by irradiation with blue light. Lethal cells showed no fluorescence. The RPE-explants were incubated with 3 mM calcein-AM in PBS (-) for 15 min at room temperature. The viable-lethal analysis was performed with a fluorescence microscopy (Eclipse Ti-E, Nikon, Japan) and a FITC-filter (excitation wavelength: 465-495 nm, dichroic for 505 nm, barrier filter at 515-555 nm).

The calcein-fluorescence representation enabled a binary evaluation. In case of a visible SRT-induced damage the lesion in this study is evaluated with 1 and in case of no damage with 0. A visible damage is defined by a group of three lethal RPE-cells, which appear black in the fluorescence microscopy.

The RPE-damage thresholds were determined by the representation of the binary evaluation in a Probit-Plot. The calculation was performed with Origin 2018. The Probit-fit provided the irradiation threshold (ED_{50} -value). An ED_{50} of an irradiation value meant, that in 50 percent of the cases the treatment with this irradiation value showed a lesion at the RPE.

3. RESULTS AND DISCUSSION

The IMF of the experimental SRT laser is between 1.3 and 1.5 for all measured parameters from $2\mu\text{s}$ with $20\mu\text{J}$ to $20\mu\text{s}$ with $420\mu\text{J}$.

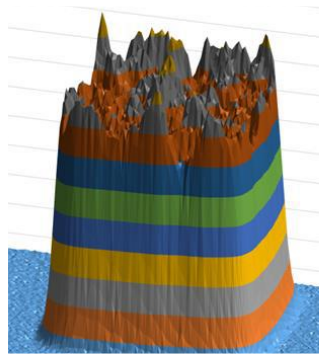


Figure 2: Intensity profile of a laser pulse with a laser pulse duration of $10\mu\text{s}$ with a pulse energy of $20\mu\text{J}$. The shown intensity profile is captured with an magnification of 9,81.

In this study 10 porcine eyes could be analysed, where 1000 lesions were set in total. Each laser pulse duration counts 200 lesions. The binary evaluation resulted in 632 visible RPE-damages (2 μ s: 85/200, 6 μ s: 131/200, 10 μ s: 125/200, 14 μ s: 144/200 and 20 μ s: 147/200). The calcein-AM assay shows the applied SRT-pattern clearly. Directly adjacent RPE-cells and surrounding regions show green fluorescence which confirms the viability of the untreated RPE. Treated spots with SRT showed a non-fluorescence which validated the RPE-cells as dead in a defined area. The applied pattern is exemplary shown in Fig. 3a. Marker and SRT-lesions differ distinctly. It becomes clear that the laser pulse durations and laser pulse energies induces visible damage thresholds at the RPE (Fig. 3b).

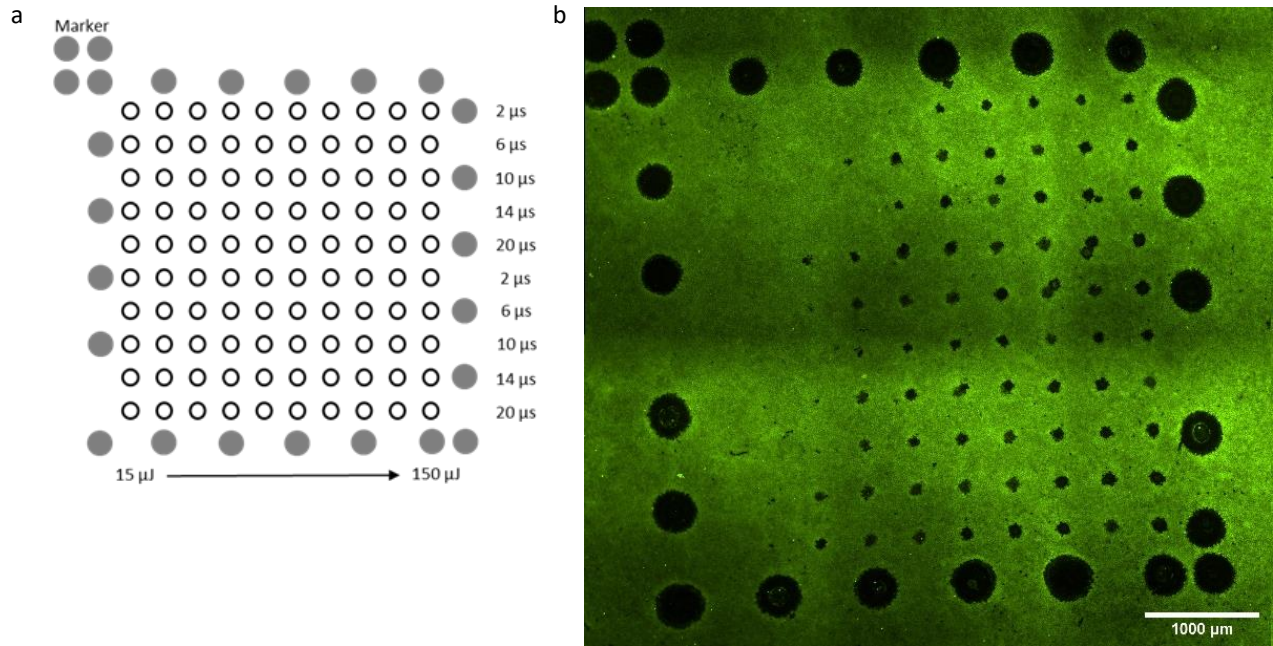


Figure 3: a) Scheme of the applied laser pattern where marker are set for orientation. For the treatment lesions each laser pulse duration is applied twice where every single spot has a different laser pulse energy. Laser pulse energy between 15 μ J and 150 μ J are used. b) Calcein image presenting the applied pattern. Dead cells show non-fluorescence and appear black, while untreated cells fluoresce in the green spectral range. Marker lesions are differentiate clearly from the treatment lesions. Visible damages appear with increasing laser pulse energy. The lowest laser pulse energy causes no damages

With the lowest laser energy of 15 μ J it is not possible to damage the RPE cell layer. For 2 μ s, first damages are obtained with laser pulse energies of 19 μ J. A laser pulse duration of 10 μ s and 14 μ s requires an energy of 29 μ J. Treatment with 14 μ s and 20 μ s requires 41 μ J and 30 μ J respectively. Feasible thresholds cannot be seen in the second half of the pattern at 6, 10, 14 and 20 μ s between the first and second visible lesion. The squared core of the laser fibre is projected occasionally as 120x120 squared lesion at the RPE (Fig. 4a). The use of the eye model provides the detection of definite transitions in the cell damage between different laser pulse energies as shown exemplary in Fig. 4b. This is the same position as in Fig. 3b in the second line of lesions for 6 μ s. A cell damage defined by 3 dead cells is clearly observed at 29 μ J for 6 μ s. With an energy of 37 μ J a considerably greater cell damage has been achieved.

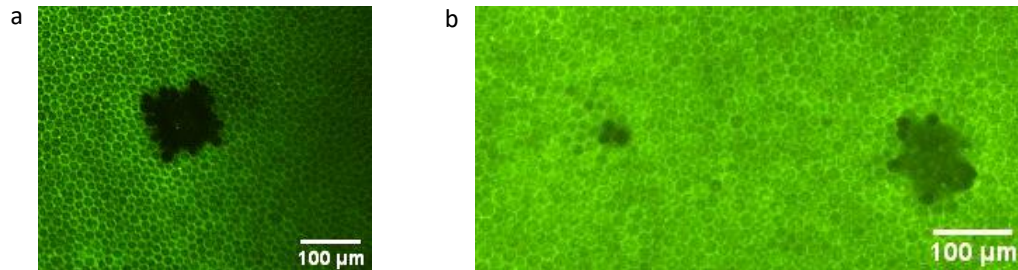


Figure 4: a) Squared damage of the laser radiation with the size of 120x120 µm. b) Transition from a cell damage of three dead cells for the laser parameter 6 µs and 29 µJ to a great cell damage for 6 µs and 37 µJ. Dead cells show no fluorescence.

With the assumption of the projected squared core fibre of 120x120 µm at the RPE, the Probit-Plot results in the following ED-values shown in Table 1. The ED-values are presented as average radiant exposure and peak radiant exposure where the latter one considers the IMF of 1,3. The minor variability of the ED₅₀-values infers a consistent pigmentation in the RPE-choroid-sclera explants. It can be assumed that the porcine eyes are adequate prepared and cultivated. Further the eye model does not lead to an additional RPE-cell loss during the trial period and an observed RPE-cell damage can be considered as a laser-induced damage.

Table 1: ED₅₀-values for different laser pulse durations presented as average radiant exposure and peak radiant exposure (IMF = 1,3).

	Laser pulse duration [µs]	Average radiant exposure [mJ/cm ²]	Peak radiant exposure [mJ/cm ²]
ED₅₀	2	179,2 ± 4,1	233,0 ± 5,3
	6	242,6 ± 5,7	315,4 ± 7,4
	10	255,3 ± 7,4	332,0 ± 9,6
	14	289,5 ± 7,9	376,4 ± 10,3
	20	299,4 ± 11,9	389,2 ± 15,5

4. CONCLUSION AND OUTLOOK

Task of this study was to establish a model which enables the investigation of damage thresholds on RPE after µs laser exposure. The preservation of the RPE-choroid-sclera explants allowed a long term work flow without losing the viability of the RPE-cells. The experimental SRT laser system proved to induce a defined RPE-damage and is therefore appropriate for laser effect on cellular level. The IMF of 1,3 is accurate for a safety treatment and unwanted effects can be neglected. For the future a narrow range of laser pulse duration and energy has to be analysed to determine the absolute threshold and further in-vivo experiments can be planned. Detailed investigation towards possible thermal non-selective damage of the surrounding tissue should be done with specific histologic examinations.

ACKNOWLEDGEMENT

The authors like to thank Meridian AG Thun: Eric Odenheimer and Heidelberg Engineering: Ralf Kessler for the valuable working together and the inspiring discussions.

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