Functional Evaluation Using Multifocal Electroretinogram After Selective Retina Therapy With a Microsecond-Pulsed Laser

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Conventional, continuous wave laser photocoagulation (cwPC) has been generally used to treat various macular diseases, including diabetic macular edema (DME), exudative AMD, and central serous chorioretinopathy (CSC).1-3 When laser light is irradiated on the retina, it is mostly absorbed by the retinal pigment epithelium (RPE), acting as the heat source in this case. From here the thermal energy is conducted from the targeted lesion toward the adjunct retinal tissue and choroid, and results in irreversible thermal damage if the temperature rise exceeds the damage threshold temperature.4,5 The results in ophthalmoscopically visible gray-to-white appearing lesions caused by increased light scattering of the tissue after thermal denaturation.5,6 Although the technique finds widespread clinical use, macular irradiation using conventional laser photocoagulation is associated with a risk of scotoma caused by extended thermal damage.6,7 It might be ideal that the laser treatment for macular lesions lying close to or within the foveal avascular zone lacks of collateral thermal damage in the neural retina.8

Selective retina therapy (SRT) was introduced to limit the induced damage to solely the RPE, and thus eliminates the risk of laser-induced scotoma caused by excessive thermal damage to the surrounding healthy neural retina and choroid, an overview on the methods, techniques, and results is presented in the literature.9 Selective retina therapy has been performed to treat macular diseases based on the mechanism that regeneration of RPE layer can be achieved in the healing phase after the degradation of dysfunctional RPE.8-10 Selective retina therapy generally uses repetitively pulsed-laser irradiation with microsecond pulse durations, unlike conventional laser photocoagulation, which employs a continuous wave (cw) laser beam for durations of typically 20 to 200 ms. Previous studies showed that the spatial extent of elevated temperature was indeed strongly reduced compared with cwPC when the multiple repetitive laser pulses in a train of 30 pulses were delivered, and also avoids potentially strong disruptive effects associated with shorter nanosecond pulse durations.11,12

In contrast to thermal effects after cwPC, the mechanism of selective RPE damage originates from microbubbles forming at the intracellular melanosomes after the temperature exceeds the vaporization threshold at their surfaces.9,11 The multiple small and short living microbubbles transiently increase the cell

PURPOSE. To evaluate the changes of retinal function with multifocal electroretinogram (mfERG), and estimate the association between functional and structural changes after selective retina therapy (SRT) with microsecond-pulsed laser in comparison to continuous wave laser photocoagulation (cwPC).

METHODS. Selective retina therapy and cwPC were applied with 10 × 10 shots and 1/2 lesion-width on the retina in the right and left eyes of 20 healthy Chinchilla Bastard rabbits, respectively. Optical coherence tomography (OCT), fundus fluorescein angiography (FFA), and mfERG were performed before, and on days 1, 7, and 30 after both laser treatments. The mean ratios of amplitudes and implicit times of N1 and P1 from eight hexagons covering laser-treated retinal lesions/total retina were measured. Histology was obtained after killing three rabbits at each time period to observe the anatomic changes after both laser treatments.

RESULTS. The mean ratios of amplitudes of N1 and P1 in SRT lesions did not change significantly for 30 days after laser treatment. Only subtle reductions of the mean ratios of N1 and P1 amplitudes on day 1, thereafter the amplitudes showed the trend to recover toward baseline values. Histology and OCT revealed temporary and reversible morphologic changes after SRT, which restored to normal within 1 month. However, the mean ratios of N1 amplitudes on days 7 and 30 (P = 0.010, P < 0.001, respectively), and P1 amplitudes on days 7 and 30 (P < 0.001, P < 0.001, respectively) declined significantly in cwPC lesions compared with baseline. Disorganization and atrophic changes were identified on histology and OCT after cwPC.

CONCLUSIONS. The results suggest that SRT preserved retinal function as well as anatomical structure after treatment.

Keywords: selective retina therapy (SRT), multifocal electroretinogram (mfERG), continuous wave laser photoagulation (cwPC)
volume, and thus thermomechanically disrupt the cellular membrane.\textsuperscript{13} This sort of damage induces ophthalmoscopically invisible, but angiographically visible spots on the fundus owing to the breakdown of the chorioretinal barrier.\textsuperscript{9,11} The transit region from thermal to thermomechanical RPE damage is found between 10- and 50-\mu s durations.\textsuperscript{14}

In order to avoid too high laser exposure leading to coalescing microbubbles with the risk of extended thermomechanical damage to adjacent retinal tissue and choroid, an automatic real-time feedback dosimetry tool is demanded in order to treat close above the RPE damage threshold. Therefore, an optoacoustic (OA) technique measuring bubble-related pressure transients with an ultrasonic microphone embedded in the contact lens was proofed to correlate with the angiographic visibility of the lesions, when introducing a suitable threshold value.\textsuperscript{15} A technique to automatically feedback control the laser emission is to ramp up the pulse energy within the burst of microsecond pulses, while detecting the onset of microbubbles for every single pulse.\textsuperscript{16} As soon as microbubbles are found the laser automatically ceases the delivery of further pulses. In that work, we used an optical feedback technique, which evaluates changes in the laser light reflected back from the retina upon the onset of microbubbles.\textsuperscript{16} The light is measured with photodiodes integrated into the laser slit lamp, the technique is called reflectometry. It is proved as suitable technique in combination with an automatic ramping function in previous study.\textsuperscript{17} The quantified feedback values from reflectometry were corresponded with OA device when stepwise increments of pulsed irradiation were delivered during SRT application (Fig. 1A).

Reflectometry as well as OA device might be regarded as an authentic and useful dosimetry tool that can control energy delivery during SRT. The selectivity and safety of reflectometry as a dosimetry tool for SRT were shown in a previous study.\textsuperscript{17} Therefore, real-time automated reflectometry was used as a...
dosimetry tool in the present study to improve the accuracy and safety of SRT.

Structural restoration of both the RPE and photoreceptor cells after SRT has been proved using histologic findings and OCT images in previous studies.5,8,18,19 However, the preservation or restoration of retinal function after SRT, as well as morphological restoration, should be considered. To our knowledge, no study has yet evaluated retinal function after SRT, except for a previous pilot study that used microperimetry after SRT in patients with macular diseases.10 In the present study, the changes of retinal function from the laser-treated lesions in rabbit eyes were evaluated for 1 month after microsecond-pulsed SRT controlled by reflectometry and conventional continuous wave laser photocoagulation (cwPC) using multifocal electroretinogram (mfERG) over time. Furthermore, changes of retinal function and anatomical structure were compared after SRT.

**METHODS**

**Animals**

The study was approved by the by the Soonchunhyang University Animal institutional review board, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 20 healthy Chinchilla Bastard rabbits were used for the present study, and were anesthetized with Zoletil 50 (125 mg zolazepam and 125 mg tiletamine hydrochloride; Vibra, Carros, France; 0.2 mg/kg of body weight) and Rompun (2% xylazine hydrochloride; Bayer Animal Health, Leverkusen, Germany; 5 mg/kg of body weight) administered intramuscularly 10 minutes before all laser treatments and examinations. Pupillary dilation was achieved with 0.5% phenylephrine hydrochloride and 0.5% tropicamide (Mydrip-P; Santen, Osaka, Japan). To prevent unexpected movement, the animals were placed in a special holding system, which allowed movements in all directions. Laser irradiation was performed using an ophthalmoscopic contact lens (Ocular Mainster OMRA-S; Ocular Instruments, Bellevue, WA) that focused the laser on the rabbit fundus. The contact lens was placed on the mydriatic eye using 0.3% hypromelloses (GenTeal; Novartis, Basel, Switzerland), and laser treatment was performed. The experiments were conducted at the same time of day.

**Laser Settings**

Irradiation with SRT was performed in the right eyes of rabbits using a prototype device of a Q-switched Nd:YLF laser system. The laser consists a diode excited Nd:YLF crystal with intracavity frequency conversion to a laser wavelength of 527 nm. The pulse duration was set to 1.7 μs, and the repetition rate to 100 Hz. The maximal numbers of pulses in a single burst was limited to 30 pulses (Fig. 1B).

An optical system was attached to a slit lamp, and created the desired laser spot with an aerial diameter of 200 μm. On the rabbit fundus, the diameter was 133 μm because of 33% optical demagnification. To determine the maximal laser energy to retinal tissue, three to four test shots were applied on the inferior peripheral retina in the initial three rabbits (Rabbit number [No.] 1–3). The maximal pulse energy that could be delivered in a single burst was determined as the highest energy to induce ophthalmoscopically invisible burns after the test shots. In general, the maximal pulse energy in each spot was set between 30 and 35 μJ (216 and 252 mJ/cm²) depending on individual rabbits. After the determination of maximal pulse energy, the energy of laser irradiation was controlled by real-time reflectometry, which was used as an automated feedback dosimetry in the present study. The first pulse energy is commenced at 10% of the maximal pulse energy. Thereafter, the next pulse energy is increased by 3.1% in each subsequent pulse. When laser pulse energy increased, the feedback values from retinal tissue were obtained using reflectometry (Fig. 1B). The “bubble set point” was established when the exponential rise in feedback values was detected with reflectometry. The laser energy at the bubble set point could be considered as the energy, which induced selective RPE damage. Hence, laser irradiation was automatically ceased within consecutive 30 shots when the feedback values were reached the preset bubble set point (Fig. 1C).

On the other hand, irradiation with cwPC was carried out using a PASCAL Streamline system (Topcon Medical Laser Systems, Inc., Santa Clara, CA, USA) delivering laser irradiation 532 nm in wavelength to the left eyes of rabbits. The spot size was 200 μm in the air, thus 133 μm on the retina, the duration of irradiation 20 msec, the laser power 125 mW, and the total energy 7.2 J/cm² in each laser spot.

Both eyes of rabbits were enrolled for this study. The aim of the study was to observe the consecutive changes after SRT and cwPC over time, rather than direct comparison of the effects between two different laser irradiation systems at each period. In addition, both eyes were used to irradiate SRT and cwPC on the identical location of the rabbit eyes. It is thought that enrollment of both eyes were not contrary to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, because laser-treated retinal lesion was locally limited area on visual streak and didn’t result in serious loss of visual function.

**Laser Application**

Irradiation with SRT was conducted in the right eye. Laser lesions were made on the retina 2-disc diameters inferior to the optic nerve head, because this area has been regarded as visual streak, corresponding to the macula of the human eye. The visual streak is a horizontal band located below the optic nerve head, and it is characterized by high density of cone cell and ganglion cell distribution.20,21 Initially, nine ophthalmoscopically visible marking spots (in a 3 × 3 pattern) were made by cwPC to prevent uneven patterning of SRT lesions, because SRT lesions were ophthalmoscopically invisible during laser application. Thereafter, a total of 91 SRT lesions were made among the marking spots at 1/2 lesion-width intervals (Fig. 2A).

Irradiation with cwPC was applied in the left eye. A total of 100 shots (in a 10 × 10 pattern) were given at 1/2 lesion-width intervals in the same areas in which SRT lesions were created in the right eyes (Fig. 2B).

**Fundus Photography and Fundus Fluorescein Angiography (FFA)**

Fundus photographs of both eyes of rabbits were taken before, and on days 1, 7, and 30 after both laser treatments. Fundus color photographs were obtained with Vx-10 fundus camera (Kowa Company Ltd., Nagoya, Japan). Fundus fluorescein angiographic images were recorded using a confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph 2; Heidelberg Engineering, Heidelberg, Germany) before, and on days 1, 7, and 30 after both laser treatments to evaluate changes of the angiographic visibility of laser-treated lesions over time. Angiographic images were obtained after injection of 0.3 mL of 10% fluorescein sodium (Fluorescite; Alcon Laboratories, Inc., Fort Worth, TX) into the
ear vein of a rabbit. After dye injection, photographs were taken every 30 seconds for 5 minutes.

**Spectral-Domain Optical Coherence Tomography (SD-OCT)**

Spectral-domain (SD)-OCT images were acquired using a combined SD-OCT and scanning laser ophthalmoscope (SLO) imaging system (Spectralis OCT; Heidelberg Engineering). Dense single raster horizontal scan was performed on laser-treated lesion to obtain the cross-sectional images of retinal tissue. Scan length was 9 mm, covering a field of 30° horizontally, and every sectional image was acquired after averaging of 20 scans to improve image quality. At least three raster scans were taken to ensure good sectional images. Spectral-domain OCT was also performed before, and on days 1, 7, and 30 after both laser treatments to evaluate the changes of anatomical structure of the retina over time.

**Histology**

Sagittal sections including the optic nerve head and the visual streak were prepared to observe morphological changes after treatment with either laser. Three rabbits were killed at each time period by injection of an overdose of the anesthetic cocktail. Both eyeballs were enucleated and immersed in 20 mL solution composed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for fixation at 4°C for 3 days. After removal of the anterior segment of the eyeball, the posterior eyecup was placed in fixative solution for 1 day. The area of interest on the posterior eyecup was carefully dissected, and placed in fresh fixative solution. After washing in phosphate buffer, each preparation was embedded in paraffin at the melting temperature (56°C). Paraffin-embedded tissue preparation was sectioned at 4-μm thickness using a microtome, and the section was stained with toluidine blue.

**Multifocal ERG Recording**

Multifocal ERG responses were recorded using Visual Evoked Response Imaging System (VERIS Science 6.4.1; Electro-Diagnostic Imaging, Inc., Redwood City, CA, USA). All recordings were performed in the same examination room under artificial room light (28 cd/m²). Rabbits were kept in the room light for 1 hour prior to examination. After pupil dilation with 0.5% phenylephrine hydrochloride and 0.5% tropicamide, recording was performed under anesthesia. Visual stimuli were displayed on a 1.5-inch fundus/stimulation camera (Electro-Diagnostic Imaging, Inc.), and the stimulus was consisted of a picture with a total of 61 equally sized hexagons, covering 30° of the visual field (Fig. 3). The fundus was visualized using an infrared fundus/stimulation camera during mfERG recording. Stimuli were provided after positioning the optic nerve head at 12 o’clock position of the recording area under infrared fundus/stimulation camera to control that the same location in rabbit eyes was consistently stimulated at each time period (Fig. 3A). In addition, fundus visualization using this camera was able to check the unfavorable movement of the eye or the stimulation camera during mfERG recording.

An ERG-jet contact lens electrode (LKC Technologies, Inc., Gaithersburg, MD, USA) was applied onto the eye of rabbit after lubrication with 0.3% hyromellose. A reference electrode was placed on one ear, and a ground electrode on the other ear. The signal gain was 50,000, and the filtering range between 10 and 300 Hz. Illumination flickered between light and dark according to a pseudorandom 75-Hz binary m-sequence with the mean stimulus luminance of 16.6 cd/m² and the contrast of 99.3%. The recording time was 13.65 sec/segment, and a total of 16 segments were repeated to improve the test accuracy.

The first-order kernels were obtained from eight hexagons covering laser-treated lesions after overlying fundus and hexagon images (Fig. 3). The amplitudes and implicit times of the first-order kernels were measured. Then, the N1 and P1 amplitudes and implicit times from the eight hexagons were averaged to allow statistical analysis. To reduce the variability of repeated mfERG recordings, the ratios of laser-treated hexagons over untreated entire retina (laser-treated retina/total retina) were analyzed at each time period. The mfERG recordings were performed before, and on days 1, 7, and 30 after both laser treatments to evaluate functional changes over time in the laser-treated retinal lesions. Due to being killed for the histologic study, the mfERG recordings were achieved for 20 rabbits before treatments, 20 rabbits on day 1, 17 rabbits on day 7, and 14 rabbits on day 30 after treatments.

**Statistical Analysis**

Statistical analysis was performed using the SPSS software (version 18.0 for Windows; SPSS, Inc., Chicago, IL, USA). The mean ratios of amplitudes and implicit times of N1 and P1 from the laser-treated eight hexagons over untreated entire retina at each time period were compared with baselines. The two-tailed Student’s t-test was used to compare the mfERG parameters among time periods. In addition, the Bonferroni correction was applied to reduce the type I error introduced by multiple comparisons. A probability value of 0.0167 was
RESULTS

Fundus Photography and Fundus Fluorescein Angiography

Fundus color photographs taken on day 1 after SRT and cwPC were represented on Figure 2 in the right and left eyes of an identical rabbit (Rabbit No. 10), respectively. On fundus photograph, only nine marking spots made using cwPC were visible in the right eye, meanwhile 91 SRT spots were invisible (Fig. 2C). On the other hand, all of 100 cwPC spots were visible with gray-to-white color in the left eye (Fig. 2D).

The angiographic visibilities of SRT and cwPC lesions were investigated using FFA at each time period. The consecutive changes of FFA are shown in Figure 4 after application of SRT and cwPC in an identical rabbit (Rabbit No. 11). On day 1 after laser application, FFA detected hyperfluorescence of all laser-treated lesions including SRT lesions and cwPC marking spots in the right eye (Fig. 4). Hyperfluorescence of SRT lesions in right eye was barely evident on day 7 (Fig. 4), and had disappeared on SRT lesions on day 30 (Fig. 4).

Hyperfluorescence was also exhibited by all cwPC lesions in the left eye on day 1 (Fig. 4), and it was still observed on cwPC lesions on day 7 after laser application (Fig. 4). However, hypofluorescence was evident on cwPC lesion on day 30 (Fig. 4).

Multifocal ERG

The results of mfERG recording revealed functional changes in laser-treated retinal lesions after SRT and cwPC. The consecutive changes of the first-order kernels from mfERG are shown in Figure 5 for 30 days after application of SRT and cwPC in an identical rabbit (Rabbit No. 15). The changes of mfERG responses obtained from eight hexagons covering laser-treated lesions were also represented after averaging of the responses (Fig. 5). Subtle reduction of the averaged amplitude was identified on day 1 after SRT compared with baseline. Thus, the amplitude tends to increase on days 7 and 30. The averaged amplitude after cwPC continued to decline for 30 days (Fig. 5).

No significant changes of the mean ratios of laser-treated/total retina in N1 amplitudes of SRT lesions were evident on days 1 (N = 20), 7 (N = 17), and 30 (N = 14) after laser treatment, compared with baseline (Fig. 6A). Additionally, the mean ratios of P1 amplitudes of SRT lesions were not altered significantly on days 1, 7, and 30 compared with baseline (Fig. 6B). On a closer view, subtle reductions of the mean N1 and P1 amplitudes were identified on day 1 after SRT compared with baseline (Figs. 6A, 6B). The N1 and P1

considered to indicate statistical significance after the Bonferroni correction.
amplitudes showed a trend to increase until day 30 (Figs. 6A, B). The N1 and P1 amplitudes showed similar changes after SRT application.

The mean ratios of N1 amplitude of cwPC lesions decreased on day 1 after laser treatment, however this value did not differ significantly from baseline. The N1 amplitude of cwPC lesions fell significantly on day 7 after treatment ($P = 0.010$), and further on day 30, also significantly lower than baseline ($P < 0.001$; Fig. 6A). The mean ratios of P1 amplitude of cwPC lesions significantly declined on day 7 compared with baseline ($P < 0.001$), and further on day 30 ($P < 0.001$; Fig. 6B).

The mean ratio of implicit times of N1 and P1 did not differ significantly from baseline at any period after SRT and cwPC (Figs. 6C, D).

SD-OCT

Series of the horizontal OCT images were acquired to represent the morphological changes after SRT and cwPC. On day 1 after laser treatment, SRT lesions were identified between marking spots made by cwPC. Dark columns throughout the inner segment/outer segment (IS/OS) junction line were evident on the OCT images obtained on day 1 after SRT (Fig. 7A). The overlying retina was left unaffected in

Figure 4. Consecutive changes of FFA findings after SRT and cwPC from Rabbit No. 11. Hyperfluorescence decreased gradually after SRT. Hyperfluorescence was observed on days 1 and 7, while hypofluorescence was evident on day 30 after cwPC.

Figure 5. Consecutive changes of mfERG traces and averaged responses after SRT and cwPC from Rabbit No. 15. Subtle reduction of the averaged amplitude was identified on day 1 after SRT compared with baseline. Thus, the amplitude showed a tendency to increase on days 7 and 30. The averaged amplitude continued to decline for 30 days after cwPC.
spite of SRT irradiation. The dark columns of SRT lesion were disappeared on day 7, and elevation of the RPE layer was evident only on the OCT images (Fig. 7B). On day 30, SRT lesions had been restored to the normal morphological structure, except for slight irregularity of the RPE layers in several lesions (Fig. 7C).

The OCT images of cwPC lesions followed a different pattern from SRT lesions. Irreversible changes were identified FIGURE 7. Consecutive changes of OCT images after SRT and cwPC. Selective retina therapy lesions (arrowheads) were identified between marking spots by cwPC (arrows; A–C). Dark columns throughout the IS/OS junction line were noted on day 1 after SRT (A), but disappeared on day 7 (B). Restoration to normal structure was evident on day 30 (C). However, dark columns were seen throughout all retinal layers on day 1 after cwPC (D). Disorganization and generalized atrophy were continued on days 7 (E) and 30 (F).

FIGURE 6. Consecutive changes of the mean ratios of mfERG parameters from laser-treated hexagons over entire retina after SRT and cwPC. The mean ratio of N1 (A) and P1 (B) amplitudes of SRT lesions were not changed significantly for 30 days. Subtle reductions of N1 and P1 amplitudes were observed on day 1. However, the mean ratios of N1 (A) and P1 (B) amplitudes from cwPC lesions decreased significantly on days 7 and 30 compared with baseline values. No significant changes of the mean ratios of N1 (C) and P1 (D) implicit times were noted. The asterisks (*) indicate a significant difference (P < 0.0167; Student’s t-test with the Bonferroni correction). Error bars = standard errors.
after cwPC application. Upward protrusions of the IS/OS junction lines were observed in cwPC lesion on day 1 after laser treatment. Dark columns throughout all retinal layers were also noted on the OCT images (Fig. 7D). Disorganization of the entire retinal lamellar architecture was evident was observed on day 7 after cwPC (Fig. 7E). On day 30, the generalized atrophic changes and disorganization in cwPC lesions progressed compared with the laser-treated lesion on day 7 (Fig. 7F).

**Histology**

Microscopic analysis of chorioretinal sections revealed the changes of the features of laser-treated retinal lesions. Selective retina therapy lesions were identified between pairs of marking spots applied using cwPC on day 1 (Fig. 8A). The RPE layer of SRT lesions exhibited increased irregularity. The OSs of photoreceptor cells were relaxed, and part of the ISs were involved. The OSs were variable in thickness and oblique in orientation. On day 7 after SRT, the ISs of photoreceptor cells had regained the normal appearance, but elongation of the OSs was still maintained (Fig. 8B). Photoreceptor cells in SRT lesions regained the normal lamellar structure of the retina on day 30 (Fig. 8C).

Histologic findings after cwPC represented irreversible changes, as the OCT images. Full-thickness damage was evident in cwPC lesions on day 1 after laser application (Fig. 8D). Disorganization was observed from the photoreceptor cell layer to the retinal nerve fiber layer. The inner and outer nuclear layer (INL, ONL) exhibited pyknosis and reduced numbers of nuclei. Cellular disorganization progressed with more pyknotic nuclei in the INL, ONL, and photoreceptor cell layer on day 7 (Fig. 8E). The densities of both the ISs/OSs decreased. In addition, the RPE became condensed and hyperpigmented. On day 30 after creating of cwPC lesions, loss of arrangement on the full-thickness retina was evident, accompanied by irregularly distributed nuclei (Fig. 8F). However, relatively less disorganized photoreceptor cell layers were still identified between atrophied retinal lesions by cwPC (Figs. 8E, 8F; arrows) despite 7 or 30 days after laser application.

**Discussion**

The concept of RPE specific therapy is targeting RPE selectively without irreversible thermal damage to overlying retinal tissue including photoreceptor cells. To confine laser energy within the RPE layer, laser irradiation should be delivered with a pulse duration shorter than the time needed for heat to diffuse toward surrounding tissue; the time is so called “thermal relaxation time.” If the laser pulses are irradiated with pulse duration shorter than the thermal relaxation time of RPE cell, selectively confined high temperature is obtained and selective RPE damage can be achieved.

Previous studies sought to develop different kinds of laser application methods using various pulse durations to target the RPE selectively in the absence of collateral thermal damage to adjacent healthy retinal tissue. Of the various pulse durations tested, a recent study recommended the use of microsecond pulse duration for SRT to retain selectivity and prevent photodisruption effect of the overlying retina. The onset of bubble formation was correlated with the pulse energy delivered when laser irradiation was applied with microsecond duration, rather than nanosecond. Also, laser irradiation with microsecond duration was concentrated principally in the RPE layer according to the previous study on retinal temperature distribution after retinal laser irradiation with various durations. Based on these theories, SRT using microsecond duration with repeated pulsed irradiation was established to target the RPE cells selectively in the absence of collateral thermal damage.

Selective RPE targeting for the treatment for retinal diseases was attempted before the development of SRT. Subthreshold laser photocoagulation using microsecond-pulsed laser has been used recently for the treatment of DME, CSC, and nonexudative AMD. Subthreshold laser photocoagulation was developed to target the RPE layer, to minimize photoreceptor cell loss and to allow for the healing response. Photocoagulation with subthreshold laser energy and continuous scanning laser technique could be practically difficult to maintain the tight focusing with slit-lamp optical system. Additionally, a previous study reported that subthreshold laser photocoagulation could induce histologic changes according the duty cycles, though invisible laser spots were made.
They demonstrated that laser spots, which were invisible ophthalmologically until 2 weeks after laser treatment became visible after 4 weeks.\textsuperscript{33} Subthreshold laser photocoagulation was generally performed using reduced laser power from test shots, which was applied near the major vessels.\textsuperscript{14} By this method, selective targeting of RPE cannot be ensured and selective damaging is not monitored in real time. It is thought that SRT with real-time dosimetry can be a more developed treatment modality in aspect of safety compared with subthreshold laser photocoagulation.

The present study was performed to explore the changes of retinal function and anatomical structure after SRT using pulses 1.7 µs in duration, and to investigate the association between functional and anatomical changes. The IS/OS junction lines of photoreceptor cells were affected by SRT application as noted on OCT images taken on day 1 (Fig. 7), and temporary elongation of the OS was also observed in histologic images (Fig. 8). The mfERG data showed that the mean amplitudes of N1 and P1 were not changed significantly for 30 days after SRT (Fig. 6). However, subtle reductions of the mean amplitudes were revealed without significance on day 1 after SRT (Figs. 5, 6). It is suggested that temporary anatomical changes in SRT lesions on day 1 were adequately reflected in the mfERG responses. On day 30 after SRT, histologic and OCT findings were restored to normal structure, and the mean N1 and P1 amplitudes were also gradually increased toward baseline values. Based on the changes of OCT, histology, and mfERG findings after SRT, it can be assumed that changes of retinal function were well-associated with those in anatomical structure. Irradiation with SRT induces anatomical changes and subtle functional alterations in retinal tissue, but such changes are temporary, which can be restored within 1 month.

On the other hand, significant reduction of the mean N1 and P1 amplitudes were observed in cwPC lesions on day 7 (Fig. 6). Furthermore, the reduction of amplitudes was progressed gradually on day 30 after cwPC. It is thought that reduction of the mean N1 and P1 amplitudes after cwPC were resulted from the changes of visibility from FFA and progressive atrophic changes from histology and OCT image. The laser spots on fundus photography and FFA were enlarged and coalesced with each other after irradiation with cwPC. This consecutive change might induce gradual decrease of mfERG amplitudes over time.

Theoretically, SRT should target the RPE layer selectively. However, photoreceptor cells were also partially affected by SRT in the present study. The subthreshold laser photocoagulation as well as SRT revealed various extent of tissue damage on laser burns in previous studies, although all laser burns were equally ophthalmoscopically invisible.\textsuperscript{19,22-23} Paulus et al.\textsuperscript{22} found that the OSs of photoreceptor cells revealed temporary structural change after SRT using microsecond-pulsed laser. The OSs of photoreceptor cells exhibited an oblique orientation and marked thickness variability on day 3, but regained a normal morphology at 1 week after SRT. In addition, Framme et al.\textsuperscript{19} also reported temporary relaxation of the photoreceptor OS, which was restored to normal structure after SRT. In the present study, temporary elongation of the OS was noted in histologic findings, and dark columns in photoreceptor cell layers were also observed in OCT images taken after SRT. Nevertheless, these anatomical changes were restored to normal structure within 30 days after SRT. In mfERG results, the mean N1 and P1 amplitudes revealed subtle reduction on day 1. The amplitudes tended to increase toward baseline values on days 7 and 30, though those didn’t recovered completely. Overall changes of the mean N1 and P1 amplitudes were not statistically significant for 30 days after SRT. Therefore, it is assumed that SRT didn’t affect significantly anatomy and function of retinal tissue, and induced reversible and temporary changes. These results suggest that SRT is an acceptable method by which to treat central macula, including subfoveal region, because the risk of laser-induced scotoma caused by irreversible thermal damage can be avoided.

The morphologic appearances of in marking spots in the right (cwPC-treated) eye and cwPC lesions in the left (only cwPC-treated) eye were not completely identical in histology and OCT images at each time period. All of cwPC lesion had disorganization and loss of arrangement on full-thickness retina; however, cwPC lesions in the left eye manifested more progressive atrophic change. It can be supposed that neighboring tissue of cwPC lesions contributes the discrepancy of structural appearances between both eyes. Laser spots were coalesced with each other over time after cwPC in the left eye. Healthy or relatively less damaged retinal tissue existed besides the cwPC lesion in SRT-treated eye, on the other hand, cwPC lesions existed in succession in cwPC-treated eye. The mean ratio of mfERG amplitudes after cwPC were reached to approximately 30% of baseline values in the current study. Mean amplitudes didn’t reduce close to noise level, although full-thickness disorganization was observed in histologic findings. As seen in Figure 8, retinal tissues including less impaired photoreceptor cell layers were still identified between cwPC lesions on days 7 and 30. In addition, a part of laser-untreated healthy retinal tissue surrounding laser-treated lesion can be inevitably included for analysis when mfERG recording is performed with covering the eight hexagons on the laser-treated retina. It is thought that these less damaged retinal tissues might contribute to induce the mfERG responses in cwPC-treated eye.

Anatomical changes in retinal tissue after SRT application have been investigated in several previous studies.\textsuperscript{5,9,22,23} Selective RPE damage has been confirmed by histologic findings and OCT images.\textsuperscript{5,9,22} However, no study has yet evaluated retinal tissue functionally after SRT in an animal model. To our knowledge, this is the first study that concerned with the functional alteration using mfERG after SRT, as well as anatomical change of the retina.

In conclusion, SRT of microsecond duration featuring repeated pulses controlled by real-time automated reflectometry could preserve the retinal function after laser irradiation, as verified by the results of mfERG recording. Temporary anatomical change was observed in photoreceptor cells after SRT, and retinal function was also slightly affected. However, those were reversible and not significant changes for 1 month after SRT. Furthermore, the changes of anatomy and function of retinal tissue after SRT were associated with each other. The use of reflectometry as a dosimetry tool could avoid the irreversible thermal damage and safely control the appropriate energy delivery during SRT. Further studies are required to improve the safety of SRT by confirming the exact therapeutic window and safety range, and to verify the efficacy of SRT used to treat the patients with various macular diseases.

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