Selective Retina Therapy and Thermal Stimulation of the Retina: Different Regenerative Properties - Implications for AMD Therapy

Elisabeth Richert
University Hospital Schleswig-Holstein
Julia Papenkort
University Hospital Schleswig-Holstein
Claus von der Burchard
University Hospital Schleswig-Holstein
Alexa Klettner
University Hospital Schleswig-Holstein
Philipp Arnold
Kiel University
Ralph Lucius
Kiel University
Ralf Brinkmann
University of Lübeck
Carsten Framme
Hannover Medical School
Johann Roider
University Hospital Schleswig-Holstein
Jan Tode (jan.tode@mh-hannover.de)
Hannover Medical School

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Abstract

Background

Selective Retina Therapy (SRT) and Thermal Stimulation of the Retina (TSR) have shown therapeutic effects on Age-related Macular Degeneration (AMD) in mice. We investigate the differences between both laser modalities concerning RPE regeneration.

Methods

For PCR array, 6 eyes of apolipoprotein E and nuclear factor erythroid-derived 2- like 2 knock out mice respectively were treated by neuroretina-sparing TSR or SRT. Untreated litter mates were controls. Eyes were enucleated either 1 or 7 days after laser treatment. For morphological analysis, porcine RPE/choroid organ cultures underwent the same laser treatment and were examined by calcein vitality staining 1 h and 1, 3 or 5 days after irradiation.

Results

TSR did not induce the expression of cell-mediators connected to cell death. SRT induced necrosis associated cytokines as well as inflammation 1 but not 7 days after treatment. Morphologically, 1 hour after TSR, there was no cell damage. One and 3 days after TSR, dense chromatin and cell destruction of single cells was seen. Five days after TSR, there were signs of migration and proliferation. In contrast, one hour after SRT a defined necrotic area within the laser spot was seen. This lesion was closed over days by migration and proliferation of adjacent cells.

Conclusions

SRT induces RPE cell death, followed by regeneration within a few days, accompanied by necrosis induced inflammation, RPE proliferation and migration. TSR does not induce immediate RPE cell death; however, migration and mitosis can be seen a few days after laser irradiation, not accompanied by necrosis-associated inflammation.

Background

Age related macular degeneration (AMD) is the most common cause for legal blindness in the industrialized world (1,2). The pathogenesis of AMD is multifactorial. Altered lipid metabolism (3–5), disturbed extracellular matrix homeostasis (6–8), inflammatory processes (9–11), and altered angiogenesis (12–14) are the four major pathways of AMD pathogenesis. Metabolites and cell-waste accumulate within and adjacent to Bruch's membrane (BrM) (3), increasing BrM thickness and thereby diffusion barrier (15), inhibiting gas and nutrient exchange. Under the influence of external factors like smoking (16), Western diet (17) and oxidative stress (18,19) as well as genetic predisposition (20), inflammatory processes appear, RPE cells and, consequently, photoreceptors degenerate leading to late stage dry AMD with patchy RPE/photoreceptor atrophy, called geographic atrophy. Pro-angiogenic factors may lead to choroidal neovascularization (CNV), forming fast progressive late stage neovascular (n)AMD. Both late-stage types of AMD are followed by vision deterioration.

Currently there is no treatment for early AMD, intermediate AMD, or geographic atrophy. Only nAMD can be treated by anti-vascular endothelial growth factor (VEGF) injections, mostly on a monthly schedule (21). The need for treatment options for early and intermediate AMD is unmet.

We could show that novel laser therapies, thermal stimulation of the retina (TSR) and selective retina therapy (SRT), reduce pathologically thickened BrM and partially restore RPEs physiological morphology (22,23). Therefore, both laser therapies might be therapeutic options for early and intermediate AMD. TSR is a continuous wave laser irradiation therapy that leads to a photothermal increase of temperature to about 45°C (22). It induces no anatomical or functional damage to neuroretina. SRT is a micro pulsed laser irradiation therapy that creates a photodisruptive selective damage to RPE, leaving the neuroretina intact (23,24).

Recently, we evaluated the influence of both TSR and SRT on inflammatory mediators (25). We could show that TSR initially acts anti-inflammatory and is followed by chemotactic processes. SRT, on the other hand, initially leads to an inflammatory response, most likely linked to the necrosis of RPE, followed by mild suppression of inflammatory mediators, like complement components, after a week. This led to the hypothesis that in SRT, RPE regeneration is the consequence of selective RPE necrosis. In TSR, RPE regeneration is the consequence of delayed RPE cell death. This hypothesis is addressed here by the evaluation of cell-death linked cell-mediator expression in murine AMD models and by the evaluation of cell morphology in calcein stained porcine organ cultures.

Materials And Methods

AMD Mouse Models

Both knock out AMD mouse models, Apolipoprotein (Apo)E knock out (-/-) and Nuclear factor erythroid 2-related factor 2 (NRF2) -/- have been described in detail (22,26,27). ApoE -/-, NRF2 -/- and C57BL6/J control mice were purchased from the Jackson Laboratories (Bar Harbour, ME, USA). The homozygous genotype and screening for Crumbs homologue1 (CRB1) retinal degeneration (rd)8 mutation, known to interfere with the AMD phenotype of NRF2-/- mice (28), was confirmed by PCR from tail clips. Mice were kept on a regular 12 hours night and day cycle and fed standard murine diet and water ad libitum. All animal experiments were conducted in accordance with the EU directive 2010/63/EU for animal experiments. They were approved by the animal ethics and welfare committee (approval number: V 242-7224.121-12 (61-5/14)) located at the ministry of energy transition, agriculture, environment and rural areas in Schleswig-Holstein according to German federal and European law. Animal experiments adhere to the NIH Guide for Care and Use of Laboratory Animals.

Animal Maintenance and Anesthesia During Experiments

All examinations and laser treatments were conducted under general anesthesia, like described before (22).

Anesthetized animals were placed on a rigid examination platform and body temperature was maintained within normal limits using a heating mat. Pupils were dilated and eyes were covered with a protective moisturizing gel. After examinations, the anesthesia was antagonized, like described before (22). Anesthesia was uneventful in all mice. Animal wellbeing was evaluated by a standard score sheet and was uneventful in all included mice. After the final examination animals were euthanized by cervical dislocation at the day of enucleation under deep anesthesia.

Examinations

All examinations were conducted under general anesthesia. All mice were examined by funduscopy (MICRON III, phoenix research labs, Pleasanton, CA, USA), to assess integrity of retina, hallmarks of AMD (drusen-like retinal spots (DRS)), RPE atrophy and CNV.

Optical coherence tomography (OCT) (small animal OCT, thorlabs, Lübeck, Germany) was applied to evaluate retinal structure, confirm retinal integrity after laser treatment and to confirm CNV.

All examinations were repeated at the day of enucleation, thus 1 day or 1 week after laser treatment. Untreated controls also were examined twice, at inclusion and at enucleation day.

Laser Treatment

For both SRT and TSR a frequency doubled Neodym-Vanadate (Nd:VO_4) experimental laser (Carl Zeiss Meditec AG, Jena, Germany) with a wavelength of 532 nm was used. The light was coupled to an optical multimode fiber with a 70 x 70 µm² core profile. The laser light was applied via contact laser-injector (Phoenix) attached to the Micron III camera. The pilot laser was controlled visually via live fundus imaging. Spot size was fixed to 50 µm².

For TSR duration of irradiation was fixed to 10 ms continuous wave mode. For SRT duration of irradiation was fixed to 300 ms, pulse-duration was ~1.4 µs at 100 Hz, creating 30 pulses per spot. Comparable to our previous work (22,23), the intended effect was titrated visually by decreasing energy at the peripheral retina from a clearly visible white burn at higher energy to a barely visible spot at lower energy. The barely but instantly visible spot was classified as threshold of definite retinal burn/RPE destruction with visible neuroretinal involvement. Power was reduced by 70 % to ensure neuroretina-sparing temperature increase for TSR, or an RPE-selective laser damage for SRT respectively. The invisible 50 µm TSR/SRT laser spots were distributed uniformly across the retina at 1 spot interspot spacing to an optic disc centered approx. 50° field of view. No laser spot was applied to vasculature or the optic disc.

PCR Array

RT² profiler PCR array (Qiagen®, Frederick, Maryland, USA; Mouse Inflammatory Response & Autoimmunity; PAMM-077Z) was used to determine regulation of inflammation and cell-death related mediators of TSR or SRT treated eyes in comparison with untreated littermates in AMD mouse models. The procedure was described in detail before (25). Briefly, for RNA isolation, posterior cups were homogenized and total RNA was isolated using TRI Reagent® according to the manufacturer's instructions.

Isolated RNA was converted to cDNA using the RT^2 First Strand Kit (Qiagen). The mixture was aliquoted (25 µl) to each well of the same RT^2 Profiler PCR Array plate (96-well plate) containing the pre-dispensed gene-specific primer sets. PCR was performed using a 7500 Fast Real Time cycler (Applied Biosystems).

Qiagens online Web analysis tool (Gene globe) was used to calculate the fold change by determining the ratio of mRNA levels to control values using the Δ threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$). All data were normalized to the housekeeping genes of PAMM007Z panel (Quiagen). PCR conditions used: hold for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Porcine Organ Cultures Preparation

Fresh porcine eyes were acquired from a local abattoir. The preparation has been described in detail elsewhere (29). Briefly, the eye bulbs were cut at the limbus removing the anterior segment, including lens and vitreous body. Eyes were opened by longitudinal incisions and neuroretina was removed. The complex of RPE, BrM and choroid was removed carefully from sclera. A plastic ring-system was inserted, and the RPE/BrM/choroid complex fixed to it. Rings were placed into 12-well-culture plate and kept warm at 37°C in 1.5 ml organ culture medium (see Richert et al. (29)).

TSR and SRT in Organ Cultures

Organ cultures were placed under a slit-lamp adapted laser system in organ culture medium in 12-well plates. Organ cultures were irradiated by either TSR (100 ms duration, 200 µm spot size, power titrated to no instant cell-death and a cell death rate of ~ 2 % one day after TSR), or by SRT (300 ms duration, 100 Hz, 1.4 µs pulse duration, 200 µm spot size, energy titrated to an initial cell-death rate of 80 %). Calcein-assays were performed afterwards to confirm cell-death rates. This method was also applied to check the quality of organ cultures (30).

Calcein Assay

Calcein assays were conducted to examine integrity and vitality of RPE organ cultures. Calcein fluoresces if cleaved by active enzymes integrated into vital RPE cell membranes. Dead cells do not fluoresce, since enzymatic cleavage does not function in dead cells (31). Organ culture explants were incubated in 2 ml culture medium with 4 µg/ml Calcein at 37 °C for 45 minutes. Afterwards they were rinsed twice in phosphate buffered saline. Cell vitality was measured by fluorescence microscopy (Axiovert 100, Zeiss, Jena, Germany) at λex/λem = 497/517 nm and documented photographically.

Statistics

Gene Expression by PCR Arrays

Fold changes in gene expression for pairwise comparison using the ΔΔCT method was calculated through Qiagen® Web analysis tool and p-values were provided, at a confidence interval of 95 % and a type-1 error of 5 %.

For comparison of TSR or SRT treated to untreated eyes, one randomized eye of ApoE-/- or NRF2-/- was treated by TSR or SRT. One day or one week after treatment these eyes were compared to entirely untreated age-matched randomized control eyes of the same genotypes in groups of 6 eyes each.

Determination of Cell Size and Number by Calcein Assays

For determination of cell death and regeneration, 1 hour, 1, 3 and 5 days after TSR or SRT laser treatment, calcein assay photographs were analyzed semiautomatically by AxioVision (Zeiss, Jena, Germany). Cell size and number of vital cells within the defined 200 µm laser spot were measured and noted for statistical analysis. The median percentage of non-fluorescent area within the defined laser spot was calculated from 12 spots each.

Results

In-Vivo Imaging

All mice showed certain signs of AMD, such as drusen-like retinal spots (DRS), RPE pigmentation irregularities and mottling. CNV or geographic atrophy, as markers for late AMD, were not seen in any mouse. AMD disease grading, like explained before (28) (1= physiological retina, 2= 1-14 DRS, 3= 15-100 DRS, 4= >100 DRS, 5= any number of DRS plus signs of late AMD), revealed a mean of grade 2.6 +/- 0.6 in NRF2-/- and 1.7 +/- 0.6 in ApoE-/- mice. Laser treatment did not significantly alter the AMD grade of either NRF2 -/- or ApoE -/- mice. There were no signs of neuroretinal damage in fundus examination or in OCT after either laser treatment.

PCR-Array for the Expression Level of Inflammatory Cell Mediators Linked to Apoptosis and Necrosis

Table 1 shows the results of PCR array-based analysis of cell mediator expression. Cell-death-linked apoptotic factors, like Fas ligand (FasL), Interferon gamma (IFNg), Interleukin (IL)1 beta and IL18, as well as Nuclear factor kappa light-chain enhancer of activated B-cells (NFκb) were examined. FasL was not altered neither by TSR nor by SRT, 1 or 7 days after laser treatment. IFNg was downregulated by TSR, 1 day after treatment in ApoE-/- and upregulated 1 day after SRT in NRF2-/- mice. IL1b was downregulated 7 days after SRT in NRF2-/- mice. IL18 was not altered in either treatment. Expression of NFκb1 was increased 1 day after SRT.

Necrosis-linked factors were also examined. Complement system centered complement factor 3 (C3) expression, was increased 4-fold, 1 day after SRT in ApoE-/-. Toll-like receptors (Tlr) were increased 1 day after SRT in both models. In NRF2 -/- mice Tlr expression was decreased 1 day after TSR. Tumor necrosis factor superfamily (Tnfsf) was downregulated 1 day after TSR in ApoE -/- mice.

Table 1
The results of PCR array-based analysis of cell mediator expression.

	ApoE								NRF2							
	TSR 1d		TSR 7d		SRT 1d		SRT 7d		TSR 1d		TSR 7d		SRT 1d		SRT 7d	
prot	fold	p	fold	p	fold	p	fold	p	fold	p	fold	p	fold	p	fold	p
FasL	1.2		1.8		1.7		-2.5		-1.5		1.1		-1.3		1.3	
IFNg	**-5.1**	<0.01	1.6		-1.9		-1.3		1.1		1.5		**2.3**	<0.01	1.9	
Il1b	-1.4		1.1		3.3		-1.7		-1.8		-1.8		-5.2		**-2.0**	0.02
Il18	1.4		1.1		1.6		-1.5		-1.6		1.1		-1.1		-1.3	
NFkb1	-1.3		-1.6		**5.0**	<0.01	1.3		-1.2		1.1		1.2		-1.7	
C3	-1.9		1.1		**4.0**	0.01	-1.4		-1.2		-1.8		1.2		-3.1	
Tlr1	-1.2		1.6		2.1		-1.7		-1.7		1.1		**2.5**	0.04	-1.4	
Tlr2	-1.8		-1.1		**4.1**	<0.01	-1.3		-1.9		1.2		-1.1		-1.1	
Tlr4	-1.5		1.1		**2.8**	0.01	-1.1		**-2.0**	<0.01	-1.1		1.5		-1.5	
Tlr7	-1.3		1.6		1.9		-2.3		**-2.3**	0.05	1.7		1.2		-1.4	
Tlr9	-1.7		1.1		**4.0**	<0.01	-1.5		**-3.0**	<0.01	-1.6		-1.6		-1.4	
Tnfsf14	**-3.5**	<0.01	2.6		1.1		-3.0		1.9		2.5		-2.5		1.3	

Single Values of Examined Apoptosis and Necrosis linked Inflammatory Cell Mediators

Column 1 shows the name of the protein examined. For each genotype, x-fold expression in the treated eyes (TSR or SRT respectively) compared with untreated eyes and their p-values are given. FasL, Ifng, Il1b, Il18, Nfkb1 may be linked to apoptosis (bold frame). C3, Tlr1,2,4,7,9 and Tnfsf14 may be linked to necrosis.

Calcein Assay

In porcine organ cultures RPE cell vitality was examined by calcein assay. In addition, regeneration processes could be examined. Figure 1 displays calcein assays after TSR and after SRT at 200 µm spot-size 1 hour, 1, 3 or 5 days after laser irradiation. There was no instantly visible cell damage 1 hour after TSR. TSR-treated organ cultures showed cell mottling, condensed nuclei from day 1 (2 % cell death ± 4.3 % within the lasered area, n= 12 spots). From day 3 (mean cell damage 4.2 % ± 3.6 within the lasered area, n= 12 spots), regenerative signs, like cell migration and cell proliferation were seen. Cell replacement and lesion closure were seen in small patches across the spot. Lesion closure was achieved at day 5. SRT induced instant cell necrosis covering the whole spot, followed by proliferation and migration from day 1 after laser irradiation (mean area of cell death 60 % ± 36.5 n= 12 spots). At day three 6.7 % (± 6.5 %, n= 12 spots) of the initial spot area were not filled with new cells. Lesion closure was complete after 5 days.

Discussion

AMD is a multifactorial disease composed of an altered lipid metabolism, changed extracellular matrix, inflammatory processes and mislead angiogenesis. There is no pathogenesis-driven therapy that targets all the above-named aspects of AMD. Current therapeutic strategies aim at certain parts of AMD pathogenesis. To date, only the treatment of pathologic angiogenesis by anti-vascular endothelial growth factor (VEGF) antagonists(21) in neovascular AMD has shown great therapeutic benefit. A therapy for early or intermediate AMD has yet to be developed. We know that TSR, as well as SRT have a therapeutic effects on AMD-like alterations in AMD mouse models. Thickened BrM becomes thinner and pathologically altered RPE becomes a more physiological phenotype (22,23). BrM restructuring aims at extracellular matrix and is mediated by an increase in matrix metallo-protease (MMP) expression, especially active MMP-2 (29,32). However, RPE regeneration may have a positive influence not only on extracellular matrix.

As for inflammation, it is more likely that inflammatory processes are altered in short term due to the laser impact on the treated RPE cells. RPE cells are reduced in viability if put under constant pro-inflammatory stress (33). This condition can be found in AMD mouse models, like ApoE -/- mice (25). We could show earlier that TSR suppresses inflammatory processes one day after treatment and is followed by chemotaxis one week after laser irradiation. SRT induces inflammation instantly due to the intended necrosis. Inflammatory processes are unaltered or even suppressed one week after laser irradiation (25). A lasting therapeutic effect that derives from suppression of pro-inflammatory processes over a long time, useful for the treatment of AMD, has not been shown so far.

The effect on lipid metabolism has not been evaluated yet. It should be part of future studies to increase the understanding of the way of action of both TSR and SRT.

The effect of TSR and SRT on neovascular AMD has also not been evaluated in a translational model. We know from organ culture experiments that both TSR and SRT lead to a reduction of VEGF expression and increase of PEDF expression (29,34). However, both laser modi have shown to influence more than only one aspect of AMD pathogenesis in AMD mouse models. Both may be therapeutic options. The unsuccessful LEAD study (35), as far as the inhibition of a conversion of intermediate to late AMD is concerned, has shown that patient selection and a good understanding of the molecular mechanisms of a new treatment are crucial.

So how exactly do TSR and SRT act?

Based on findings from this paper, as well as findings from former publications (22,23,25,29,34,36), we propose the following model, as depicted in figure 2.

SRT, as expected, leads to defined necrosis of RPE (24) (see Fig. 1, 2 and Table 1). The photo-disruptive effect induces RPE cell death that does not harm neuroretina (23,37). Necrosis is accompanied by inflammation and chemotaxis to remove cell-debris (25). RPE regenerates (29) and active MMP expression is increased, leading to thinning of the pathologically thickened BrM in AMD (23,38). Restoring and rejuvenating RPE could be a useful therapeutic approach to treat intermediate AMD.

TSR (see Fig. 2), on the other hand, leads to a delayed small scale RPE cell-death (see Fig.1), followed by immediate replacement of RPE cells (34), without inflammation (25) and, like SRT, without neuroretinal damage (22). The regenerative process is accompanied by an increase in active MMP expression (34), also leading to thinning of BrM. A regenerative process of RPE cells can also be seen (22). This less invasive, less immunogenic approach might be a therapeutic means for mild AMD or even for AMD prevention.

From the presented data and from what we have known so far, one cannot decide if either TSR or SRT are the better therapeutic option for the treatment of a certain type of AMD. More needs to be known about the way of action to better attribute either laser modus to a certain

type of AMD.

The above shown data is limited by small numbers of eyes and methods to differentiate between the different regenerative properties. However, the differences between both laser modi become clearly evident. SRT leads to necrosis followed by regeneration. TSR leads to delayed RPE cell death in absence of inflammation also followed by regeneration. A presumed apoptotic process needs to be looked at closer in future studies.

Despite uncertainties concerning laser therapy for early and intermediate AMD it is reasonable to consider both SRT and TSR potential therapeutic means for AMD. Both need to be evaluated in humans. Study populations need to be selected carefully, considering the different grades and forms of AMD. TSR might be an option for early AMD with small drusen, SRT for a more advanced type that might need more therapeutic effect. Future studies, especially human studies, will have to determine the benefit of TSR and SRT for the treatment of early and intermediate AMD.

Abbreviations

AMD age-related macular degeneration

ApoE -/- apolipoprotein E knock out

BrM Bruch's Membrane

C3 complement factor 3

CNV choroidal neovascularization

CRB1 crumbs homologue gene 1

DRS drusen like retinal spots

FasL Fas ligand

IFNg interferon gamma

IL interleukin

MMP matrix metallo proteinase

nAMD neovascular age-related macular degeneration

NFκb Nuclear factor kappa light-chain enhancer of activated B-cells

NRF2 -/- nuclear factor erythroid 2-related factor 2

OCT optical coherence tomography

RPE retinal pigment epithelium

SRT Selective Retina Therapy

Tlr toll-like receptor

Tnfsf Tumor necrosis factor superfamily

TSR Thermal Stimulation of the Retina

VEGF vascular endothelial growth factor

Declarations And Acknowledgments

Ethics Approval

All animal experiments were conducted in accordance with the EU directive 2010/63/EU for animal experiments. They were approved by the animal ethics and welfare committee (approval number: V 242-7224.121-12 (61-5/14)) located at the ministry of energy transition, agriculture, environment and rural areas in Schleswig-Holstein according to German federal and European law. Animal experiments adhere to the NIH Guide for Care and Use of Laboratory Animals.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests

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Author's Contributions

ER planning experiments, conducting experiments, data evaluation, manuscript revision

JP conducting experiments, data evaluation, manuscript revision

CvB data evaluation, manuscript revision, statistics

AK manuscript revision, laboratory support, experimental support

PA manuscript revision, laboratory support, experimental support

RL manuscript revision, laboratory support, experimental support

RB manuscript revision, laser device, experimental and technical support

CF manuscript revision, data revision

JR manuscript revision, data revision

JT planning experiments, conducting experiments, data evaluation, manuscript preparation, manuscript revision, funding, correspondence

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Figures

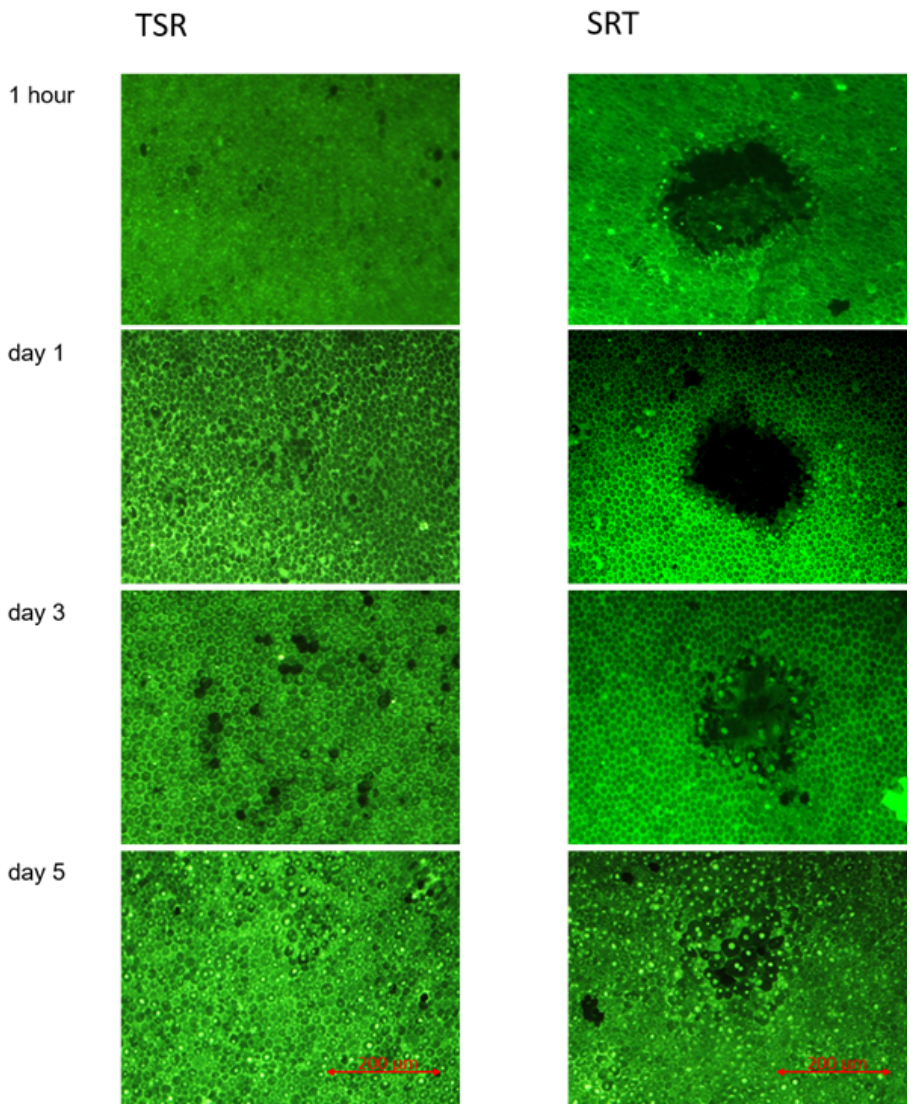

Figure 1

Representative examples of calcein assays to determine cell vitality and proliferation at the different given time points after TSR and SRT. TSR is followed by cell death and replacement from 1 to 3 days after laser irradiation. SRT induced instant RPE cell-death, followed by regenerative processes like migration and proliferation at the rim of laser lesions until lesion closure.

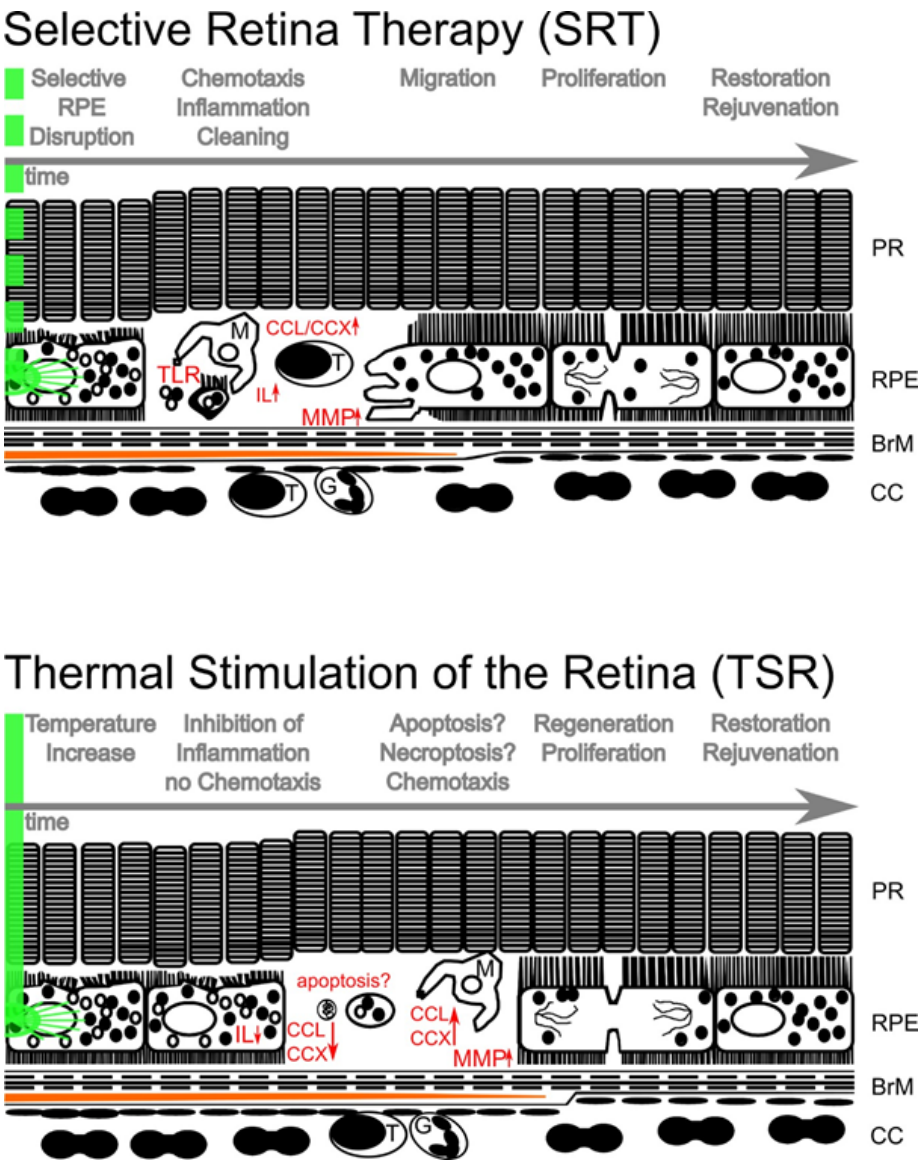

Figure 2

SRT and TSR presumed way of action SRT (above) induces initial necrosis by photodisruption of RPE. Toll-like receptors (TLR) are expressed. Microglia (M) are attracted, interleukins (IL) and chemokines (CCL/CCX) are increased in expression thereby attracting more cells of the immune system. Destroyed cells are removed and neighboring RPE starts migration and proliferation to close the lesion. Increased matrix metallo-proteases (MMP) restructure Bruch's membrane (BrM) and intra-BrM lipids (orange line) are removed. A rejuvenated, restored BrM/RPE complex is achieved thereafter. Neuroretina stays intact during this process. TSR (below) does not induce necrosis. Inflammatory processes, like interleukin expression and chemotaxis are initially suppressed. Single RPE cells die and are instantly removed. This presumably apoptotic process, since cell death and replacement are seen in absence of inflammation, is accompanied by migration and proliferation of RPE cells. MMP expression is increased leading to BrM remodeling and removal of accumulated lipids (orange line). A rejuvenated restored BrM/RPE complex is achieved without damage to neuroretina. PR (photoreceptors), G (granulocytes), T (T-lymphocytes).