

Ellex 2RT Laboratory Research

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Nanosecond Pulse Lasers for Retinal Applications.

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Background and Objectives: Thermal lasers are routinely used to treat certain retinal disorders although they cause collateral damage to photoreceptors. The current study evaluated a confined, non-conductive thermal, 3-nanosecond pulse laser in order to determine how to produce the greatest therapeutic range without causing collateral damage. Data were compared with that obtained from a standard thermal laser.

Materials and Methods: Porcine ocular explants were used; apposed neuroretina was also in place for actual laser treatment. After treatment, the retina was removed and a calcein-AM assay was used to assess retinal pigmented epithelium (RPE) cell viability in the explants. Histological methods were also employed to examine lased transverse explant sections. Three nanosecond pulse lasers with either speckle- or gaussian-beam profile were employed in the study. Comparisons were made with a 100 milliseconds continuous wave (CW) 532 nm laser. The therapeutic energy range ratio was defined as the minimum visible effect threshold (VET) versus the minimum detectable RPE kill threshold.

Results: The 3-nanosecond lasers produced markedly lower minimum RPE kill threshold levels than the CW laser (e.g., 36 mJ/cm² for speckle-beam and 89 mJ/cm² for gaussian-beam profile nanosecond lasers vs. 7,958 mJ/cm² for CW laser). VET values were also correspondingly lower for the nanosecond lasers (130 mJ/cm² for 3 nanoseconds speckle-beam and 219 mJ/cm² for gaussian-beam profile vs. 1,0346 mJ/cm² for CW laser). Thus, the therapeutic range ratios obtained with the nanosecond lasers were much more favorable than that obtained by the CW laser: 3.6:1 for the speckle-beam and 2.5:1 for the gaussian-beam profile 3-nanosecond lasers versus 1.3:1 for the CW laser.

Conclusions: Nanosecond lasers, particularly with a speckle-beam profile, provide a much wider therapeutic range of energies over which RPE treatment can be performed, without damage to the apposed retina, as compared with conventional CW lasers. These results may have important implications for the treatment of retinal disease.

ARVO 2010 Abstracts

Upregulation of MMP-2 and -9 Release by Human RPE Cells Mediates Bruch's Membrane Permeability.

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Purpose: The aging retina is characterized by accumulation of various lipid-rich extracellular matrix (ECM) deposits within Bruch's membrane. Such changes interfere with the transport functions of Bruch's membrane for the delivery of nutrients and removal of waste products to and from the RPE and retina. The production of active forms of matrix metalloproteinases (MMPs) mediates the degradation of abnormal collagens in aged and AMD affected Bruch's and

thereby rejuvenate the membrane and improve its transport characteristics. The purpose of the current study is therefore to assess the potential impacts of upregulation of MMPs release on improvement of Bruch's membrane permeability.

Methods: Human Bruch's-Choroid (hBC) membranes and human RPE-Bruch's-Choroid (RPE-BC) explants were clamped in our tailor-made organ culture system and maintained in DMEM/ Ham's F-12, supplemented with 1% FCS at physiological conditions for 1-3 days to allow equilibration. The hRBC explants were then irradiated with Ellex 2RT nanosecond laser system. Human RPE cells from primary cultures were introduced onto the hBC explants. At different time intervals the presence of MMPs in the bathing medium was determined before and after cells seeding and laser treatment by standard gelatine zymography and densitometric analyses. Permeability was studied by using the organ culture system described above with a dye or a labeled dextran tracer. The rate of diffusion across the hBC preparations was determined by using standard colorimetric analysis.

Results: A significant upregulation of activated MMPs 2&9 was observed following introduction of human primary RPE cells on the hBC explants. Transport studies show that the permeability of the hBC membranes to the labeled tracer was dramatically increased from 0.08 ± 0.021 (OD585) at 0 day (pre-seeding) to 0.43 ± 0.015 (OD585) at 3 days and 0.63 ± 0.025 (OD585) at 7 days post-seeding, a 4.98 folds and 7.33 folds increases, respectively (Mean \pm SEM, $p < 0.001$, $n=4$) following the growth of RPE cells on the hBC membrane. A similar pattern was observed on RPE-BC explants with 2RT laser injuries.

Conclusions: Enhancing the synthesis and release of active forms of MMPs 2&9 from RPE cells and entry of such active species into Bruch's allows for improvement in the transport characteristics of the membrane.

Laser-Mediated Activation of Matrix Metalloproteases (MMPs) Leading to Increased Transport Across Ageing Bruch's Membrane: Implications for Treatment of ARMD.

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Purpose: To assess the involvement of active MMP-9 in the proposed improvement of hydraulic transport across human Bruch's membrane (BM) following laser mediated retinal rejuvenation therapy.

Methods: Isolated samples of human RPE-Bruch's-choroid ($n=54$; age 23-92 yr) were mounted in Perspex tissue cassettes with a central 6mm aperture. One third of the samples (subset 1; $n=18$) were used to measure baseline hydraulic conductivity (HC) across BM following removal of RPE. The central 4mm portion of RPE of the second subset ($n=18$) was irradiated with 2RT laser (30 'burns' each resulting from a train of 50×3 ns pulses; $TRE=31.8$ J. cm^2). The third subset ($n=18$) was not irradiated to act as procedural control. The cassettes were incubated DMEM + 10% FCS for 21 days with medium changes every 3 days. The RPE monolayer from subsets 2 & 3 was then removed from underlying BM, washed with PBS and inserted into vertical Ussing chambers equipped with a hydrostatic pressure feed and associated digital manometers to follow fluid (movement through the preparation. An initial pressure head with PBS (corresponding to IOP) was applied to BM and the corresponding change in pressure profile as fluid traversed the preparation was used to calculate the basal HC of the preparation and following incubation period. At the end of the experiment, tissue samples were processed for zymography to determine the MMP profiles within the preparation.

Results: Baseline HC of Bruch's membrane in 54 donor samples (age-range 23-92 yr) showed an age-related exponential decline ($HC = 85.1 \exp(-0.065 \cdot \text{age})$) with a half-life of 11 years, compatible with previous studies. Irradiated samples (Subset 2; n=18, age-range 23-92 yr) showed a marked improvement in HC ($p < 0.05$), raising the transport curves upwards ($HC = 121.2 \exp(-0.03 \cdot \text{age})$) with an improved half-life for the decay function of 16 years. The improvement was particularly marked in the younger subset of donors. No significant change in HC was found in procedural controls. Zymographic analysis of MMP content post-incubation showed greater availability of active MMP-9 in irradiated samples, reflecting reduced porosity in this subset.

Conclusion: Modulation of the MMP system in RPE cells that leads to the release of activated MMP-9 provides one mechanism for improving the transport characteristics of ageing human BM. This mechanism appears to operate during RPE wound healing following laser insult. These results will be discussed in relation to retinal rejuvenation therapy for the management of ARMD.

ARVO 2009 Abstracts

Retinal Damage Profiles and Cellular Responses to Laser Treatment: Comparison Between a Novel, Non-Thermal Laser and a Conventional Photocoagulator

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Purpose: Thermal lasers remain the gold standard for treatment of diabetic retinopathy; however, there are many associated clinical side-effects such as the inability to treat within the foveal region due to collateral retinal damage. Ongoing clinical trials indicate that a novel nanosecond pulse laser (2RT) reduces diabetic macular edema. In the current study we compared retinal damage and cellular responses resulting from laser treatment of rats with a conventional thermal photocoagulator laser and the 2RT laser at clinically relevant energy levels.

Methods: Pigmented Dark Agouti rats were treated with approximately 100 shots of either a conventional photocoagulator laser (532nm, CW laser, Ellex Integre with 0.1sec pulse duration) or a nanosecond pulse laser (532nm, Q-switched Retinal Regeneration Therapy laser prototype, Ellex 2RT with 3 nanosecond pulse duration). Laser settings were at visible lesion threshold for the CW laser and at visible lesion threshold and below for the 2RT laser. Rats were killed at various time points following the procedure. Samples were processed for histology, immunohistochemistry, in situ biochemical assays, real-time and conventional RT-PCR and Western blotting using standard methodologies.

Results: The photocoagulator laser caused retinal lesions that were principally evident within the outer retina. These were associated with photoreceptor cell death, astrocyte and Müller cell activation, and infiltration of macrophages and neutrophils. In addition, upregulations of inflammatory cytokines, heat shock proteins, endogenous trophic factors, and matrix metalloproteinases were induced. In comparison, all of these changes were drastically attenuated when the 2RT laser was used, particularly at the sub-visual power setting, although RPE cell loss was still evident.

Conclusions: The photocoagulator laser produced marked retinal damage and cellular responses consistent with an inflammatory response to thermal injury. In contrast, the 2RT laser produced negligible retinal damage and cellular responses at clinically relevant energy levels, and only marginally greater effects at elevated energy levels. These

results show that nanosecond pulses of the 2RT laser produce an isolated insult at the level of the RPE without causing collateral damage to the neuroretina, which is a normal clinical consequence of thermal photocoagulator laser procedures. These results may have important implications for the treatment of diabetic macular edema, proliferative diabetic retinopathy and early AMD.

Effect of Sub-Threshold Laser Energy (Ellex 2RT) on the Matrix Metalloproteinase (MMP) Release Profile of RPE and the Diffusional Status of Underlying Bruch's Membrane

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Purpose: To investigate the dose-response relationship for laser (Ellex 2RT) induced changes in the MMP release profiles of human RPE cells and their effects on the diffusional status of underlying Bruch's membrane.

Methods: Human RPE-Bruch's-Choroid (RPE-BCM) explants were maintained under organ culture conditions for 3 days and were then irradiated with the Ellex 2RT nanosecond laser system, delivering energies of 0.3mJ and 0.6 mJ/pulse (total energy 120mJ/cm²) and returned to the incubator for a further 14 days. Following laser treatment, media samples were obtained every 24 hours for quantification of MMPs 2&9 by standard gelatine zymography and densitometric analyses. Permeability studies of the BCM preparation were performed in Ussing chambers by quantifying the rate of diffusion of a dye across the preparation using standard colorimetric instrumentation. Having determined basal diffusion levels, primary RPE cells were plated on BCM. At various time intervals, RPE were brushed off and diffusion rates measured.

Results: Varying laser energy was without effect on levels of Pro-MMP2 but the higher energies considerably elevated levels of Pro-MMP9. Active levels of both MMPs 2&9 showed a time dependent change with increasing levels peaking around post-laser day 7 with a subsequent decline that had not reached baseline by day 14. At their peak, active MMP 2&9 levels were 6.7 ± 2.6 and 4.37 ± 1.1 (Mean \pm SEM) fold higher than control. Transport studies performed after the seeded RPE cells had achieved confluence demonstrated much improved diffusional status of Bruch's membrane.

Conclusions: The Ellex 2RT laser system provides a facility for enhancing the synthesis and release of active forms of MMPs 2&9 from RPE cells. Entry of such active species into Bruch's allows for improvement in the transport characteristics of the membrane.

ARVO 2008 Abstracts

Hydrodynamic Status of Bruch's Membrane in Age-Related Macular Degeneration (AMD)

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Purpose: To evaluate the contribution of disease processes associated with AMD to the age-related decline in the hydraulic conductivity of Bruch's membrane.

Methods: Bruch's-choroid samples were isolated as 8mm discs from the peripheral regions of donor human eyes and mounted in purpose-built Ussing-type chambers. A hydrostatic pressure head of 14-16mmHg (IOP) was applied to the Bruch's side of the preparation and the resulting fall in pressure as fluid moved through the tissue complex was used

to calculate the hydraulic conductivity of the preparation. The data from the control population (90 eyes, age range 1-91 years) was used to obtain an age profile for changes in hydraulic conductivity. This profile was compared with data from 12 donors with AMD, age-range 64-95 years.

Results: In the peripheral fundus of normal donor eyes, hydraulic conductivity of Bruch's membrane declined exponentially with age and was described by the relationship $HC = A \times e^{-k \times \text{age}}$ where A was equal to 101.5×10^{-10} m/s/Pa and k, the decay constant was 0.0299, $r=0.82$ ($n=90$). The half-life of the decay process was calculated to be 23 years, i.e., conductivity of Bruch's membrane was halved for every 23 years of life. The minimum hydraulic conductivity (failure threshold) of Bruch's required to process fluid output from human RPE was calculated to be 0.57×10^{-10} m/s/Pa. Thus, in the normal population, the capacity for transport across Bruch's remained well above the minimum requirement over a human life-span. In fact, the trend line of the decay process would meet the failure threshold at an age of 173 years, well outside human life expectancy. The hydraulic conductivities of Bruch's from AMD donors all fell below the normal trend line with one donor (aged 86 years) crossing the failure threshold.

Conclusions: In AMD donor eyes, the age-related decline in hydraulic conductivity of Bruch's membrane is accelerated with capacity to reach failure threshold within the life span of an individual. Since these ageing changes are much more advanced at macular locations, these results provide an explanation for the higher incidence of RPE detachments in AMD patients. Moreover, the early failure in fluid transport capability is likely to provide an early insult to the RPE-photoreceptor complex that may be the basis of transition to the pathological phase of the disease process.

ARPE-19 Mediated Improvement in the Hydraulic Conductivity of Human Donor Bruch's Membrane

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Purpose: To evaluate the effect of seeding ARPE-19 cells on the hydrodynamic properties of donor human Bruch's membrane.

Methods: Bruch's-choroid samples (4-6 per donor eye) were isolated as 8mm discs from the peripheral regions of the fundus and mounted in Perspex tissue cassettes with a 4mm central aperture. Individual cassettes were then inserted into modified Ussing-type chambers and the Bruch's surface exposed to a hydrostatic pressure head of 14-16mmHg. The hydraulic conductivity of each preparation was calculated from the rate of fluid transport. The cassettes were then transferred to a Petri dish with exposed Bruch's surface facing upwards. A suspension of ARPE-19 cells was then placed on top of Bruch's membrane and after an incubation period of 24 hours, all but one of the cassettes from a given donor were rinsed and the attached ARPE-19 cells gently brushed away followed by re-evaluation of the hydraulic conductivity of the preparations. A representative cassette from each donor was processed for light microscopy.

Results: Histological examination of the preparations from donors aged 21-91 years showed that the 24-hour incubation period was sufficient to allow attachment and proliferation of ARPE cells resulting in a confluent monolayer on top of Bruch's membrane. Seeding of ARPE-19 cells onto donor Bruch's was associated with a marked improvement (3.4-fold \pm 1.4 S.D; $p<0.05$) in the hydraulic conductivity of the membrane ($n=16$; age range: 21-91 years).

Conclusions: Proliferating RPE cells have the potential to modulate the transport properties of Bruch's membrane. After treatment, the normal age-related exponentially declining curves were displaced upwards towards improved transport.

The potential for development of this technique to promote endogenous RPE migration into localised laser lesions may allow 'cleansing' of aged Bruch's membrane. This has implications for therapeutic management in age-related macular degeneration (AMD).

Sub-Threshold Q-Switched Laser (Ellex 2RT) - Induced Migration of Human RPE and Concomitant Release of Matrix Metalloproteinases (MMPs)

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Purpose: To investigate cellular and biochemical responses (wound healing and release patterns of MMPs) of human retinal pigment epithelium (RPE) associated with Q-switched laser exposure at energy levels below visual threshold.

Methods: Human RPE-Bruch's-Choroid explants (12-72 hours post-mortem) were maintained under organ culture conditions for 3-7 days and baseline MMP release patterns established. The explants were then irradiated with an Ellex 2RT nanosecond laser system (Q-switched YAG laser (532nm) delivering 3 nanosecond pulses; 400um spot size, 0.078mJ energy setting) and returned to the incubator for a further 14 days. At selected intervals, confocal laser scanning, conventional microscopy and a cell proliferation assay (BrdU) assessed RPE cellular dynamics. Following the laser treatment, media samples were obtained every 24 hours for quantification of MMPs 2 & 9 by standard gelatine zymography and densitometric analyses.

Results: Following 24 hour laser intervention, nearly 50% of the RPE cells within the treatment zone showed signs of injury. Most of the damaged RPE was characterised by some degree of cell membrane disruption whilst others showed disintegration of structure. By post-treatment days 5-7, most of the injured cells were replaced by migrating RPE cells from regions surrounding the lesion. Differential effects on the components of the MMP system accompanied laser treatment. Thus, released levels of inactive MMP-2 (pro-enzyme) were little altered over the two-week experimental period. However, inactive MMP-9 (pro-enzyme) increased 1.34-fold by day 1 to reach a 2.8-fold level by day 7 (n=5). Changes in the active forms of MMPs 2&9 were much more profound with levels increasing 6- and 5-fold above controls respectively (n=6). These high levels peaked around 5-7 days post-laser returning to baseline by about day 14.

Conclusions: The 2RT laser treatment procedure resulted in mild RPE injury with the subsequent wound healing process leading to the release of active matrix metalloproteinase enzymes. This transitory elevation of active MMPs may be beneficial in addressing the pathological alterations of Bruch's membrane and thus potentially useful for intervention in AMD before significant loss of vision has occurred.

Nanosecond Laser Pulses for Retinal Treatment

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Purpose: To evaluate an exciting new approach to retinal laser treatment using nanosecond laser pulses. Also to determine which laser pulse parameters produce the greatest therapeutic range for RPE treatment without causing collateral damage. Different 532nm nanosecond laser beam profiles and pulsing techniques were compared with the effects produced by millisecond laser pulses.

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| ELLEX 2RT™ RETINAL REJUVENATION THERAPY |

Methods: Porcine explants were used, with neuro-retina in place for laser treatment. The calcein-AM live cell assay was used to visualise RPE samples and light microscopy for retinal samples. The following 532nm lasers were used:

1. FD-YAG, 3ns pulse duration, speckle beam profile
2. FD-YAG, 3ns pulse duration, gaussian beam profile
3. Quasi-CW laser, 100ms & 10ms pulse durations, flat top profile.
4. CW laser , 100ms & 10ms pulse durations, flat top profile.

Threshold levels were determined by image analysis and therapeutic range ratio defined as minimum visible effect threshold versus minimum RPE kill threshold within the laser spot diameter.

Results: Both of the 3ns lasers produced much lower RPE kill thresholds than the CW or Quasi-CW lasers due to thermal confinement resulting from the temporal confinement of energy within nanosecond pulses. The 3ns laser with the speckle beam profile produced lower RPE kill thresholds than the 3ns laser with a gaussian beam profile due to the additional spatial confinement of energy. Using nanosecond pulses the visible effect threshold was found to be largely coincident with the RPE cell membrane rupture threshold. A therapeutic range ratio greater than 6:1 was achieved using nanosecond laser 1 while producing no discernable neuro-retinal damage.

Conclusions: Nanosecond laser pulses can provide a very wide therapeutic range of energies in which RPE treatment can be performed, without damage to the neuro-retina, using energy levels approximately 200 times less than CW or Quasi-CW photocoagulators. Nanosecond laser pulses with a fine speckle beam profile were found to be the most effective and produced a therapeutic range greater than 6:1 which to our knowledge is wider than previously reported. These results may have important implications for the treatment of early age-related macular degeneration and diabetic macular edema.



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