

CHARACTERIZATION OF RAT RETINAL RESPONSES TO A
CONVENTIONAL RETINAL LASER AND A NOVEL SHORT PULSE DURATION
LASER

O'SAM SHIBEEB
BSc MBBS

DISCIPLINE OF OPHTHALMOLOGY & VISUAL SCIENCES
SCHOOL OF MEDICINE
UNIVERSITY OF ADELAIDE

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF MASTER
OF PHILOSOPHY (OPHTHALMOLOGY)

MAY 2014

ABSTRACT

Retinal laser photocoagulation is the process whereby laser energy is directed onto the retina and converted to heat within the melanosomes of the retinal pigment epithelium (RPE). Its principle clinical purposes are to reduce macula oedema, inhibit angiogenesis, and create chorioretinal scars. The exact biological mechanisms by which destruction of RPE/photoreceptors leads to a therapeutic resolution of oedema remain to be elucidated. However, it is well documented that laser photocoagulation causes thermal injury to the overlying photoreceptors. The desire to produce a clinically effective and safer retinal laser motivated the development of a novel 3-nanosecond pulse duration laser, Retinal Regeneration Therapy (2RT; Ellex Pty. Ltd., Adelaide), which delivers approximately 0.2% of the energy per pulse compared to a conventional continuous wave photocoagulation laser. Recent laboratory and clinical research has demonstrated that the 2RT laser caused remarkably little destructive injury to the overlying photoreceptor layer, but still effectively ablated RPE cells. The retinal glial response to this laser had not been characterized. Furthermore, previously published research indicated that retinal lasers can stimulate endogenous production of neuronal survival factors, such as heat shock proteins and trophic factors, but the effect of 2RT on retinal trophic factors was unknown. Hence, this thesis was motivated by the notion that 2RT may be capable of inducing an endogenous neuroprotective response in the retina without causing collateral retinal damage.

The aims of Part I were: (1) to study the RPE/retinal damage profiles and neuronal effects of a CW laser and the short pulse duration 2RT laser; and (2) to characterize in detail the glial and inflammatory responses to the above retinal lasers.

The aims of Part II to were: (1) to examine whether multiple preconditioning pathways are activated by CW and 2RT lasers; (2) to examine whether preconditioning with either laser is neuroprotective in a rat model of retinal ganglion cell degeneration caused by calibrated optic nerve crush.

Pigmented Dark Agouti rats were used and treated with either a conventional, thermal, continuous wave (CW; 532nm, 100ms pulse duration) or a short-pulse (2RT; 532nm, Q-switched, 3ns pulse) laser. Settings were at visible threshold for the CW laser (12.7J/cm²/pulse) and at supra- and sub-visible threshold for the 2RT laser (“High”, 2RT-H, 163mJ/cm²/pulse; “Low”, 2RT-L,

109mJ/cm²/pulse). At various time points after lasering, rats were killed and analysed for histology, immunohistochemistry, RT-PCR and Western immunoblotting. In Part II, groups of rats were killed at 6 hours, 1 day and 1 week following the laser treatment. Samples were taken for immunohistochemistry, RT-PCR and Western immunoblotting. Rats were randomly assigned to one of three treatment groups: sham, CW or 2RT. For the CW and 2RT groups, 20 laser spots were applied to the mid-central retina of the right eye, while left eyes remained untreated. At 1 day or 7 days after lasering, rats in all three groups received ON crush in the right eye. The left ON remained intact. Rats were allowed to recover and killed 1 week or 2 weeks after optic nerve crush for quantification of RGCs in wholemounts.

The results of chapters 2 & 3, showed both lasers caused focal loss of RPE cells with no destruction of Bruch's Membrane; RPE cells were present at lesion sites again within 7 days of treatments. There were no obvious effects to horizontal, amacrine or ganglion cells, as defined by immunolabeling, but an activation of PKC α within bipolar cells was noted. There was little discernible damage to any cells other than the RPE with the 2RT-L treatment. The CW laser caused outer retinal lesions that were associated with photoreceptor death, astrocyte and Müller cell activation, and infiltration of macrophages and neutrophils. Furthermore, 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-threshold setting.

In chapter 4, the results showed that both the CW and 2RT lasers induced local glial cell activation and induced localized upregulations of a number of well-documented (CNTF, FGF-2 Hsp27, pAKT) or putative (cFOS, ATF-3, IL-6) RGC survival factors. However, neither laser caused sustained increases in other factors associated with neuronal preconditioning, such as BDNF, Hsp70, IGF-1, bcl-2, and nitric oxide synthase. As regards neuroprotection, analysis of the data revealed that ON crush resulted in the loss of approximately 70% of Brn3a-labelled RGCs after 1 week.

In conclusion the CW laser photocoagulation caused death of RPE cells with associated damage to the outer retina but negligible impact on the inner retina. The 2RT laser, at the lower setting, was able to selectively kill RPE cells without causing collateral damage to photoreceptors.

It was demonstrated that the CW laser produced astrocyte and Müller cell activation, and infiltration of macrophages and neutrophils. Furthermore, inflammatory cytokines, heat shock proteins, endogenous trophic factors, and matrix metalloproteinases were induced. Similar changes were observed when the 2RT laser was used, but the effects were less striking. Furthermore, both CW and 2RT lasers activated multiple preconditioning pathways but the stimulation of these pathways was insufficient to augment RGC survival after optic nerve crush.

PUBLICATIONS RELEVANT TO THIS THESIS

1. Wood JP, **Shibeeb O**, Plunkett M, Casson RJ, Chidlow G. Retinal Damage Profiles and Neuronal Effects of Laser Treatment: Comparison of a Conventional Photocoagulator and a Novel, 3Nanosecond Pulse Laser. *Invest Ophthalmol Vis Sci.* 2013; 28;54 (3):2305-18.
2. Chidlow G, **Shibeeb O**, Plunkett M, Casson RJ, Wood JP. Glial Cell and Inflammatory Responses to Retinal Laser Treatment: Comparison of a Conventional Photocoagulator and a Novel, 3Nanosecond Pulse Laser, *Invest Ophthalmol Vis Sci.* 2013; 28;54 (3):2319-32.
3. **Shibeeb O**, Wood J, Casson RJ, P Chidlow G. Effects of a Conventional Photocoagulator and a 3Nanosecond Pulse Laser on Preconditioning Responses and Retinal Ganglion Cell Survival after Optic Nerve Crush. *Exp Eye Res.* 2014;127:77-90.

PRESENTATIONS RELEVANT TO THIS THESIS

1. **Shibeeb O**. Preconditioning with a Novel Retinal Laser (2RT) in Experimental Glaucoma. **2013 RANZCO SA Branch Conference**; Clinical and Experimental Glaucoma in SA and Beyond, Barossa Valley, South Australia
2. **Shibeeb O**. Effects of Retinal Lasers on Retinal Ganglion Cell Survival After Optic Nerve Crush Presented at *ARVO 2012, Ft. Lauderdale, U.S.A.*
3. **Shibeeb O**. Rat Retinal Responses to Treatment with a Photocoagulation Laser and a Nanosecond pulse Laser. *Clinical Experimental Ophthalmology*, 2011; 39 (Suppl 1). Presented at *RANZCO Scientific Congress 2011*, Canberra, Australia.

ACKNOWLEDGEMENT

Firstly, I would like to thank my supervisor Professor Robert Casson, for so generously accepting me as a Master student and for his constant encouragement and his friendship. It has been a great privilege to have been supervised by Professor Casson. I am grateful for his guidance and the support. His belief in my ability to undertake this work created the opportunity for me to undertake this study.

My greatest acknowledgment and appreciation must go to my associated supervisors Drs Glyn Chidlow and John Wood for their constant support, constantly discussed ideas, co-authored manuscripts, and friendship. In particular my gratitude is extended to Dr Chidlow for the countless hours of support and providing help on demand. In addition his constant encouragement and belief in me was paramount in my success, without which, the undertaking of this project would have been difficult to achieve.

Special thanks to Mark Daymon for his knowledge and expertise, technical assistant with my laboratory work, patience, good humour and friendship. Mark has been extremely helpful in running difficult experiments despite of his own commitments.

I would also like to acknowledge the Discipline of Ophthalmology and Visual Sciences and the South Australian Institute of Ophthalmology for allowing me to undertake this project. Special thanks to Julia Winnick for her assistance with administration and support.

I would like to thank Malcolm Plunket from Ellex Pty Ltd, (Ellex Adelaide) for partially funding this project, and for financial assistance to travel to the 2011 RANZCO Scientific Congress in Canberra and ARVO 2012 in Ft. Lauderdale to present the findings of this research.

Finally I would like to extend my thanks to my wife, Meriam for her constant support, encouragement, advice and much needed distraction.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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The experiments in this thesis were performed at the Ophthalmic Research Laboratory in the Hanson Institute, Adelaide.

MAY 2014

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ABBREVIATIONS

| | |
|--------------|--|
| ATP | Adenosine triphosphate |
| Bcl-2 | B-cell lymphoma protein |
| BDNF | Brain-derived neurotrophic factor |
| cAMP | Adenosine-3'5' -cyclic monophosphate |
| cDNA | Complementary deoxyribonucleic acid |
| CNS | Central nervous system |
| CNTF | Ciliary neurotrophic factor |
| CW | Continuous wave |
| eNOS | Endothelial nitric oxide synthase |
| FGF-2 | Basic fibroblast growth factor |
| GAPDH | Glyceraldehyde phosphate dehydrogenase |
| GCL | Ganglion cell layer |
| GDGF | Glial-derived neurotrophic factor |
| GFAP | glial fibrillary acidic protein |
| Hsps | Heat shock proteins |
| ILM | Inner limiting membrane |
| INL | Inner nuclear layer |
| (ICAM)-1 | Intercellular adhesion molecule |
| IL-1 β | Interleukin-1 β |
| iNOS | Inducible nitric oxide synthase |
| IOP | Intraocular pressure |
| IPC | Ischaemic preconditioning |
| IPL | Inner plexiform layer |
| JNK | JUN N-terminal kinase |

| | |
|--------------|---|
| MHC | Major histocompatibility |
| MMP-9 | Matrix metalloproteinase-9 |
| Nd:YAG | Neodymium-doped yttrium aluminium garnet |
| NFL | Nerve fibre layer |
| NT-3 | Neurotrophin-3 |
| NT-4 | Neurotrophin-4 |
| NGF | Nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| OLM | Outer limiting membrane |
| ONL | Outer nuclear layer |
| ON | Optic nerve |
| OPL | Outer plexiform layer |
| PBS | Phosphate buffered saline |
| PKC α | Protein kinase C α |
| PCNA | Proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| ROS | Reactive oxygen species |
| RPE | Retinal pigment epithelial |
| 2RT-H | Retinal Rejuvenation Therapy-High |
| 2RT- L | Retinal Rejuvenation Therapy-Low |
| RT-PCR | Reverse-transcription polymerisation chain reaction |
| RTK | Tyrosine kinase receptor |
| TF | Transcription factor |
| TNF α | Tumor necrosis factor- α |
| Trk | Protein tyrosine kinase |