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

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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 PKCα 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.

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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.

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TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

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-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IOP	Intraocular pressure
IPC	Ischaemic preconditioning
IPL	Inner plexiform layer
JNK	JUN N-terminal kinase

MHC	Major histocompatability
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-mthyl-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
PKCα	Protein kinase Cα
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
TNFα	Tumor necrosis factor-α
Trk	Protein tyrosine kinase