Dynamic, *in vivo*, real-time detection of retinal oxidative status in a model of elevated intraocular pressure using a novel, reversibly responsive, profluorescent nitroxide probe.

Cassie L Rayner[1], Glen A Gole[2], Steven E Bottle[3], Nigel L Barnett[1, 4, 5]

[1] Queensland Eye Institute, South Brisbane, Queensland, Australia.

[2] Department of Paediatrics & Child Health, University of Queensland, Brisbane, Queensland, Australia.

[3] ARC Centre of Excellence for Free Radical Chemistry School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Queensland, Australia.

[4] The University of Queensland, UQ Centre for Clinical Research, Herston, Queensland, Australia.

[5] School of Biomedical Sciences, Queensland University of Technology, Brisbane, Queensland, Australia.

*Corresponding author:

Nigel L Barnett

Queensland Eye Institute,

140 Melbourne St, South Brisbane, QLD 4101, Australia.

Email: nigel.barnett@qei.org.au

Tel: +61-7-3239 5021
Fax: +61-7-3844 1914

Abstract

Changes to the redox status of biological systems have been implicated in the pathogenesis of a wide variety of disorders including cancer, ischemic-reperfusion (I/R) injury and neurodegeneration. In times of metabolic stress e.g. ischemia / reperfusion, reactive oxygen species (ROS) production overwhelms the intrinsic antioxidant capacity of the cell, damaging vital cellular components. The ability to quantify ROS changes *in vivo,* is therefore essential to understanding their biological role. Here we evaluate the suitability of a novel reversible profluorescent probe containing a redox-sensitive nitroxide moiety (methyl ester tetraethylrhodamine nitroxide, ME-TRN), as an *in vivo,* real-time reporter of retinal oxidative status. The reversible nature of the probe's response offers the unique advantage of being able to monitor redox changes in both oxidizing and reducing directions in real time. After intravitreal administration of the ME-TRN probe, we induced ROS production in rat retina using an established model of complete, acute retinal ischemia followed by reperfusion. After restoration of blood flow, retinas were imaged using a Micron III rodent fundus fluorescence imaging system, to quantify the redox-response of the probe. Fluorescent intensity declined during the first 60 minutes of reperfusion. The ROS-induced change in probe fluorescence was ameliorated with the retinal antioxidant, lutein. Fluorescence intensity in non-ischemic eyes did not change significantly. This new probe and imaging technology provide a reversible and real-time response to oxidative changes and may allow the *in vivo* testing of antioxidant therapies of potential benefit to a range of diseases linked to oxidative stress.

Key words: oxidative stress, retina, fluorescent probe, nitroxide, reactive oxygen species, ischemia, reperfusion

Abbreviations:
DMSO: Dimethyl sulfoxide
ERG: Electroretinogram
GFAP: Glial fibrillary acidic protein
IBA-1: Ionized calcium binding adaptor molecule 1
IOP: Intraocular pressure
I/R: Ischemia-reperfusion
ME-TRN: Methyl ester tetraethylrhodamine nitroxide
OPs: Oscillatory potentials
PFN: Profluorescent nitroxides
RGC: Retinal ganglion cell
ROS: Reactive oxygen species
AMD: Age-related macular degeneration

The accumulation of free radicals (reactive oxygen species, ROS), has been implicated in numerous neurodegenerative diseases, including Parkinson's disease (Ray et al., 2014), Alzheimer's disease (Aliev et al., 2013), cardiac diseases (Schwarz et al., 2014) and the major degenerative visual diseases such as glaucoma (Almasieh et al., 2012; Chrysostomou et al., 2013; Yuki et al., 2011), diabetic retinopathy (Wilkinson-Berka et al., 2013) and age-related macular degeneration (AMD) (Seo et al., 2012). Neurodegeneration is defined by the progressive loss of specific neuronal cell populations, with extensive evidence verifying oxidative stress a contributing factor to disease pathogenesis (Barnham et al., 2004; Scherz-Shouval and Elazar, 2011). ROS are natural by-products of cellular metabolism essential in cell signalling and homeostasis, however when cellular ROS production overwhelms the intrinsic antioxidant capacity, they become extremely damaging to vital cellular components which can lead to irreversible changes and cell death (Tezel, 2006). In response to this imbalance in the cellular redox environment, i.e. oxidative stress, cells respond by activating various defence mechanisms (Scherz-Shouval and Elazar, 2007) such as up-regulation of antioxidants and/or the removal of damaged proteins and organelles by autophagy; these assist cells in restoring homeostasis (Hamanaka and Chandel, 2010; Kiffin et al., 2006; Lemasters, 2005; Scherz-Shouval and Elazar, 2007). Accumulating data has implicated mitochondria as the main source for regulation of autophagy by ROS (Azad et al., 2009; Chen et al., 2007; Hamanaka and Chandel, 2010; Scherz-Shouval et al., 2007). Significantly, whilst retinal photoreceptors have the highest density of mitochondria of all central nervous system neurones (Kageyama and Wong-Riley, 1984), retinal ganglion cells are also particularly susceptible to mitochondrial dysfunction, which has critical importance in the initiation of glaucoma and subsequent vision loss (Chrysostomou et al., 2013; Osborne and del Olmo-Aguado, 2013).

Retinal ischemic-reperfusion (I/R) injury induced by transient elevation of intraocular pressure (IOP) in animal models, results in necrosis and apoptosis of cells in both the ganglion cell layer and inner nuclear layer (Hughes, 1991; Kuroiwa et al., 1998; Li et al., 2009; Oharazawa et al., 2010). Excessive elevation of IOP impairs blood flow dynamics in the retina and the optic nerve head, reducing the delivery of energy and nutrients required for cell survival, thus rendering them susceptible to additional insults (Osborne, 2008). Extensive evidence for this pathological mechanism has lead to the vascular theory of glaucoma (Arend et al., 2004; Chung et al., 1999; Hall et al., 2001; Mitchell et al., 2005). ROS production is a complex, dynamic phenomenon occurring during and after a period of cellular energy

deprivation, e.g. acute I/R injury, predisposing the retina to oxidative damage (Abramov et al., 2007; Li et al., 2009). The ability to detect and quantify ROS *in vivo* and in real-time, is therefore essential to understanding their biological roles.

Fluorescent detection or imaging with redox-responsive probes is a potentially powerful approach because of its merits of high sensitivity, easy visualization, simple operation, high spatial resolution in microscopic imaging techniques and most importantly *in vivo* application (Fernandez-Suarez and Ting, 2008; Wen-Xue and Xu, 2012; Xu et al., 2013). Previously, fluorescent techniques largely involved the *irreversible* reaction of a non-fluorescent probe molecule with a radical of interest to produce a detectable fluorescent product (Halliwell and Whiteman, 2004; Morrow et al., 2010; Wardman, 2007). Such 'one-way' detection methodologies can have limitations, as they are not able to respond to dynamic changes to the cellular redox environment. With the chemistry of the probe response being irreversible, such techniques therefore cannot be used to identify the potential therapeutic benefit of antioxidant intervention following the induction of a pro-oxidant stress. This ultimately prompted the development of reversible 'two-way' probes, with stable nitroxide radicals having significant potential in this regard (Morrow et al., 2010).

Nitroxides have previously been employed to probe various biophysical and biochemical processes involving oxidative stress (Ahn et al., 2012; Hirosawa et al., 2012; Mitchell et al., 2001; Wang et al., 2013), due to their high scavenger ability, reactivity to ROS (Ahn et al., 2012) and on the basis of their metabolism to reduced hydroxylamine (Morrow et al., 2010). Various cellular redox processes are capable of mediating the conversion between the reduced hydroxylamine and the oxidized nitroxide species and hence the ratio of these two states is indicative of the overall "reducing capacity", or redox environment, of the cell (Belkin et al., 1987; Morrow et al., 2010; Swartz, 1987, 1990). Covalently linking a nitroxide moiety to a fluorescent structure possessing excitation and emission profiles of biological relevance (Morrow et al., 2010), e.g. rhodamine, efficiently quenches the excited state that leads to fluorescence (Ahn et al., 2012; Blough and Simpson, 1988; Green et al., 1990). The removal of the nitroxide free radical through a one-electron reduction to the non-radical hydroxylamine, removes this quenching effect resulting in the restoration of the typical fluorescence of the fluorophore (Blough and Simpson, 1988). As the response of the nitroxide is reversible and is reflected in changes in the fluorescence emission, this enables a unique investigative tool with the potential to provide real-time insight into diseases of

oxidative stress in the eye (Morrow et al., 2010). These probes have been described as profluorescent nitroxides (PFN) as the fluorescence is switched on through metabolic or chemical processes akin to prodrugs.

We have developed a novel, reversible, PFN probe (methyl ester tetraethylrhodamine nitroxide; ME-TRN), based on the rhodamine class of fluorescent dyes. We chose this structure due to its excellent fluorescent quantum yields, high solubility and stability in water and most importantly for its selective accumulation by mitochondria in living cells (Johnson et al., 1980; Morrow et al., 2010). Here we evaluate the suitability of an ME-TRN probe as an *in vivo*, real-time reporter of retinal oxidative status. The utility of PFN probes to quantify oxidative status in isolated cells by flow cytometry and fluorescence imaging has been previously demonstrated (Ahn et al., 2012; Morrow et al., 2010) and to translate these findings to the retina, *in vivo*, we used an established model of complete, acute retinal ischemia followed by reperfusion. The generation of ROS following the ischemic insult provides a known, *in vivo*, pro-oxidant condition, upon which we based our initial investigations. The known antioxidant and free radical scavenger, lutein, has previously shown to protect macula and photoreceptors from phototoxicity and oxidative injury (Alves-Rodrigues and Shao, 2004; Chucair et al., 2007; Li et al., 2009) and more recently the inner retinal neurons from ischemic-reperfusion challenge, possibly by reducing oxidative stress (Dilsiz et al., 2006; Li et al., 2009). Based on these findings, lutein is considered an effective antioxidant. Accordingly, we used lutein to verify the fluorescent redox response of our probe. A validated technique for the measurement of retinal oxidative status *in vivo* could have a major impact on the ability to assess other putative neuroprotective antioxidants for the treatment of glaucoma and other neurodegenerative diseases.

2. Materials and Methods

2.1 Animals and Treatments

Albino Sprague-Dawley rats (female, ~250 g) obtained from the Animal Resources Centre (Canning Vale, WA, Australia) at 8 weeks of age, were housed at Herston Medical Research Centre Animal Facility (Royal Brisbane & Women's Hospital, Australia). Animals were maintained in temperature and humidity controlled rooms (~37 ˚C and 60 – 70 %

respectively), with food and water available *ad libitum*. A 12:12 hr light/dark cycle (lights on at 7 a.m.) was used, with illumination provided by overhead fluorescent white lights.

Animals were divided into four treatment groups for I/R studies: (i) control/non-ischemic (n= 9), (ii) acute I/R injury (n= 6), (iii) lutein (n= 7) or (iv) acute I/R injury + lutein (n= 6). Each group received an intravitreal injection of ME-TRN probe, prior to I/R or non-ischemic treatment. Lutein or vehicle 10 % dimethyl sulfoxide (DMSO) was administered by intraperitoneal injection 1 hour prior to I/R (see preparation below).

Animals prepared for ERG and immunohistochemistry (n= 3) were administered ME-TRN probe to one eye with the contralateral eye serving as a control for comparison purposes. Control eyes received a 2 µl vehicle injection of 10 % DMSO in injectable saline.

Experiments were conducted in accordance with the 'Animal Care and Protection Act (QLD) 2001', 'The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

2.2 ME-TRN administration

Animals were anaesthetized by intraperitoneal injection (i.p.) of 75 mg/kg ketamine and 15 mg/kg xylazine. Pupils were dilated with 1 % tropicamide and 2.5 % phenylepherine (Bausch and Lomb, Tampa, FL), and corneas anaesthetized with 0.5 % tetracaine hydrochloride (Chauvin Pharmaceuticals, Kingston-Upon-Thames, UK). ME-TRN (2 µl of 5×10^{-5} M in 10 % DMSO / injectable water) was injected into the vitreous of both eyes using a 10 µL Hamilton syringe coupled to a 26 gauge needle, to give a final concentration of 2 µM. Intravitreal injections were made posterior to the superior limbus at a 45° angle, preventing the probe from being injected into the lens.

The probe was permitted to disperse throughout the vitreous and accumulate in the retina for 30 mins prior to recording pre-ischemic retinal images using a Micron III rodent fundus imaging system (Phoenix Research Labs, Pleasanton, CA, USA) equipped with rhodamine filters (Ex 556 / Em 590 nm). Pre-ischemic fundus images provided base line intensity measures for comparison of ME-TRN fluorescent signal change induced by superoxide production during reperfusion and for the potential ameliorative effect of antioxidant

intervention. Data are expressed as fluorescence intensity as a percentage of pre-insult values. Fluorescent intensity was subsequently measured 5, 10, 15, 30, 45 and 60 mins post ischemic insult during the reperfusion phase.

To ensure similar fundus images were captured pre- and post I/R injury, optic nerve and major retinal blood vessel locations were clearly indicated and pre-ischaemic camera and animal stage positioning were fixed; animals were simply lifted away from the Micron III to allow correct repositioning following treatment. Bright field images (consistent illumination settings) were captured at each time point to verify fundus location prior to capturing fluorescent images. For each experimental animal, consistent excitation illumination levels and gain settings were used to acquire the fluorescent intensity images and were maintained for each image captured throughout the treatment period.

2.3 Image Analysis

Colour fluorescent fundus images were imported into Image J (National Institutes of Health, Bethesda, MD, USA) and converted to 8-bit greyscale. Average pixel intensity across the isolated circular whole fundus image was calculated to provide fluorescence intensity values for each time point. To account for possible variability of absolute fundus fluorescence levels between animals due to probe diffusion or other confounding factors, data are presented as the change in fluorescent intensity at each time point during reperfusion as a percentage of the corresponding pre-ischemia value (mean ± SEM).

2.4 Antioxidant Preparation

Lutein (0.05 mg/ml) was prepared in injectable saline containing 10 % DMSO and administered (0.2 mg/kg i.p.) 1 hour prior to I/R insult (Li et al., 2009).

2.5 Evaluating ME-TRN Toxicity on Retinal Tissue using Electroretinography (ERG)

Full-field ERGs were recorded as previously described (Moxon-Lester et al., 2009) 1 day (n= 3) and 8 days (n= 3) following ME-TRN administration to examine potential toxicity of the probe to the retina. ME-TRN probe was injected into the vitreous (as above) of the left eye, with the contralateral eye serving as the control. Briefly, rats were dark-adapted overnight and prepared for recording under dim red light using LED illumination (λ_{max} = 650 nm). After anaesthesia and mydriasis, reference electrodes were placed on each ear and a grounding electrode positioned subcutaneously on the back. A platinum wire recording

electrode was positioned on each cornea, which was kept moist with GenTeal gel (Novartis Pharmaceuticals, NSW, Australia). Body temperature was maintained at 37 °C with an electric animal heating blanket. Rats were then placed in a custom-designed ganzfeld and subjected to the ERG flash stimuli. ERGs were recorded simultaneously from both eyes. Scotopic responses recorded at 1.2 log cd.s.m^{-2} were elicited with a photographic flash unit (Metz mecablitz 60CT4, Zirndorf, Germany). An average of 2-3 flashes were recorded with an interstimulus interval of 300 sec to allow complete recovery of b-wave amplitudes. Responses were amplified and recorded with a bioamplifier/analogue-to-digital converter (Powerlab/4ST, ADInstruments, Castle Hill, Australia), band-pass-filtered between 0.3 and 1000 Hz, and digitized at 4 kHz.

2.6 Acute Retinal Ischemia by Elevation of IOP

Unilateral retinal ischemia was induced as previously described (Holcombe et al., 2008; Moxon-Lester et al., 2009). Briefly, anaesthetized animals were immobilized by resting the front teeth over a horizontal stabilizing bar and securing the skull with adjustable rods inserted in the bony external ear canals. The anterior chamber was cannulated with a 30-gauge needle attached to a reservoir containing 0.9 % NaCl. A micromanipulator was used to insert the needle into the anterior chamber parallel to the iris plane at the 12 o'clock position. Intraocular pressure was increased to 120 mmHg by elevation of the reservoir to 163 cm. Ocular ischemia was confirmed by the blanching of the iris and interruption of the retinal circulation. Leakage from the initial ME-TRN injection site was rarely seen, however if it transpired, animals were immediately removed from the study as complete ischaemia could not be verified. After 60 mins of elevated IOP, removal of the cannula allowed reperfusion, generating superoxide radicals upon restoration of blood flow. During reperfusion, retinal fluorescence generated by the ME-TRN probe was imaged at 5, 10, 15, 30, 45 and 60 mins. Corneal hydration was maintained throughout.

2.7 Evaluating Retinal Damage using Immunohistochemistry

Rats were euthanized with Lethabarb (200 mg/kg i.p., Virbac, NSW, Australia) 8 days following ME-TRN administration and I/R. Eyes were enucleated and fixed in 10 % neutral buffered formalin for 2 hours at room temperature. Posterior eye cups were cryoprotected with 30 % sucrose before mounting and freezing in OCT (Tissue Tek, ProSciTech, QLD, Australia). Transverse sections (10 μm) were cut using a cryostat and maintained at -20 °C until required for immunostaining, using standard methods. Slides were incubated overnight

at room temperature with polyclonal rabbit anti-Glial Fibrillary Acidic Protein (GFAP; 1:1000, DakoCytomation, Glostrup, Denmark), or polyclonal rabbit anti-Ionized Calcium Binding Adaptor molecule 1 (IBA-1; 1:2000, Wako, Osaka, Japan). Immunolabelling was visualized with Fluorolink Cy2 labelled goat-anti-rabbit IgG (Amersham BioSciences, Buckinghamshire, UK,) incubated for 90 mins at room temperature. Images were viewed with an Olympus, BX41TF microscope (Tokyo, Japan) equipped with epifluorescence and captured with an Olympus DP70 camera. Images were imported into Adobe Photoshop CS5 for minor editing of contrast and sharpness.

2.8 Data Analysis

ERG and fluorescent intensity responses are expressed as the mean ± SEM. Statistical comparisons were made using a non-parametric Wilcoxon matched-pairs signed rank test for ERGs.

Following confirmation with a D'Agostino-Pearson omnibus test that the fluorescence intensity data from each treatment group were normally distributed, the data were analyzed and compared with a linear model using R software (The R Foundation for Statistical Computing). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 ME-TRN uptake in rat retina

For the ME-TRN probe to be considered a suitable tool for the real-time measurement of retinal oxidative status *in vivo*, uptake into retina cells is necessary. Figure 1 shows a transverse section of rat retina confirming that the ME-TRN probe is selectively accumulated by retinal neurons following intravitreal administration. These data confirm that the observed changes in fundus fluorescence represent the response of retinal cells to changes in their cellular environment.

3.2 ME-TRN administration and retinal function

The ERG, commonly used to assess the functional integrity of the retina, allows for early detection and monitoring of adverse drug effects on the visual pathways. The a-wave is the

response generated by photoreceptors (Penn and Hagins, 1969) and the b-wave is predominantly generated by the depolarization of the ON bipolar cells (Bush and Sieving, 1996; Hood and Birch, 1996; Robson and Frishman, 1995; Robson and Frishman, 1998; Sieving et al., 1994). The oscillatory potentials (OPs) arise from activity in the inner retina, including a major contribution from amacrine cells (Wachtmeister and Dowling, 1978) Prior to employing and evaluating our ME-TRN probe as a real-time reporter of retinal oxidative status *in vivo*, we initially examined its 'short and long term' toxicity to retinal tissues and their function.

ERG responses were recorded from ME-TRN and vehicle treated eyes at 1 day (short term) and 8 days (long term) post administration (Fig. 2). Vehicle and ME-TRN treated eyes showed no significant difference in a-wave amplitude (1 day: 345 ± 45 µV and 400 ± 77 µV respectively; p= 0.75, and 8 days: 408 ± 36 µV and 475 ± 84 µV respectively; p= 0.50) or b-wave amplitude (1 day: 948 ± 131 µV and 1033 ± 150 µV respectively; p= 0.75, and 8 days: 975 ± 82 µV and 1073 ± 128 µV respectively; p= 0.75) (Fig. 2b). Similarly, neither a-wave nor b-wave implicit times were affected by ME-TRN injection (a-wave – 1 day: 7.25 ± 0.29 ms and 7.17 ± 0.22 ms, p$\geq$ 0.99; 8 days: 7.67 ± 0.30 ms and 7.58 ± 0.08 ms, p$\geq$ 0.99; b-wave – 1 day: 57.08 ± 7.83 ms and 55.67 ± 7.41 ms, p= 0.50; 8 days: 46.00 ± 0.76 ms and 50.42 ± 3.36 ms, p= 0.25, for control and ME-TRN treated eyes respectively). No significant change in OP peak amplitude was observed (1 day: 69 ± 11 µV and 72 ± 12 µV, p$\geq$ 0.99; 8 days: 61 ± 10 µV and 63 ± 10 µV, p= 0.99, for control and ME-TRN treated eyes respectively). These data suggest that the ME-TRN probe has no detrimental effect on retinal function and can be used at concentrations described.

3.3 Immunohistochemistry

To investigate possible toxic effects of the ME-TRN probe on the retina, we analyzed glial cell activation as the hallmark of reactive gliosis and neuroinflammation with GFAP (macroglia) and IBA-1 (microglia/macrophages) staining (Fig. 3).

In the healthy retina, GFAP is the main intermediate filament protein in astrocytes, and has limited or no expression in Müller cells. Eight days after the intravitreal injection of ME-TRN, GFAP immunoreactivity was still restricted to the astrocytes present in the nerve fibre layer. As reported previously (Larsen and Osborne, 1996)**,** an acute I/R injury induced a drastic upregulation in GFAP immunoreactivity, significantly enhancing GFAP distribution

to glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells.

In response to various stimuli, microglia transform into activated forms that can be distinguished by their morphology and antigenicity (Streit et al., 1988). When activated, the morphology of microglia include a rounded nucleus, abundant cytoplasm and short processes (Ito et al., 1998). IBA-1 labelled microglia (Fig. 3) in post I/R retina exhibit a morphology indicative of activation. However, no evidence of microglial activation could be seen in ME-TRN treated retinas, which were qualitatively similar to control retinas.

3.4 Quantification of Fluorescence Intensity following I/R Injury

The reperfusion phase following an acute ischemic insult generates ROS that become highly damaging to cellular components when ROS overwhelms the cell's intrinsic antioxidant capacity. The complete acute I/R rat model therefore provided a known, *in vivo*, pro-oxidant condition, upon which we based our investigations.

The initial assessment of ME-TRN fluorescent intensity in non-ischemic control eyes, demonstrated that the probe's fluorescence in the retina was relatively stable over a 120 min treatment period (60 mins of sham ischemia plus 60 mins imaging during sham reperfusion) (Fig. 4). Figure 5 shows that the production of superoxide upon restoration of blood flow following the ischemic insult resulted in a significant ($F_{(5,30)}$ = 2.864, p = 0.031) decrease in fundus fluorescence intensity over the 60 mins of reperfusion (white triangles) when compared to the non-ischemic, control group (white circles), with significant reductions seen at 10, 15, 30, 45 and 60 mins reperfusion; (72.17 ± 6.47 % vs 84.50 ± 4.18 % at 5 mins reperfusion vs pre-ischemia; p= 0.0577, 63.33 ± 4.09 % vs 79.50 ± 2.91 % at 10 mins; p= 0.0075, 60.33 ± 4.42 % vs 76.88 ± 4.84 % at 15 mins; p= 0.0102, 60.67 ± 4.79 % vs 74.75 ± 3.86 % at 30 mins; p= 0.0099, 52.20 ± 6.11 % vs 72.50 ± 4.35 % at 45 mins; p= 0.004, and 47.17 ± 5.59 % vs 68.71 ± 5.42 % at 60 mins; p= 0.0013, respectively). The absence of reducing equivalents and the presence of oxygen resulted in the equilibrium between the oxidized nitroxide molecule and the reduced hydroxylamine being pushed towards the more oxidized form of the species (Morrow et al., 2010). This oxidized form is highly unstable and rapidly converts back to the non-fluorescent nitroxide radical state, resulting in the decrease in fluorescence we observed within this treatment group.

In non-ischemic eyes, the intraperitoneal administration of the antioxidant lutein had no significant effect on ME-TRN retinal fluorescence throughout the treatment period (Fig. 5, black circles) when compared with the non-ischemic control group ($F_{(5,36)} = 1.354$, p = 0.245). This confirmed that any effects seen within the I/R + lutein treatment group were the result of the antioxidative properties of lutein reducing overall oxidative stress levels. Antioxidant intervention (black triangles) successfully ameliorated the decrease in retinal fluorescence induced by I/R injury ($F_{(5,30)} = 5.706$, p= 0.001), with significantly increased fluorescence observed at 5, 10 and 15 mins into the reperfusion phase; (72.17 ± 6.47 % vs 90.50 ± 5.86 % at 5 mins; p= 0.026, 63.33 ± 4.09 % vs 86.33 ± 7.61 % at 10 mins; p= 0.0047, 60.33 ± 4.42 % vs 83.71 ± 2.25% at 15 mins; p= 0.0044, 60.67 ± 4.79 % vs 71.00 ± 3.97 % at 30 mins; p= 0.1573, 52.20 ± 6.11 % vs 64.00 ± 5.56 % at 45 mins; p= 0.1691, and 47.17 ± 5.59 % vs 57.83 ± 1.83 % at 60 mins; p= 0.2783 for I/R and I/R + lutein treatment groups respectively).

4. Discussion

Pro-oxidants are recognized as having a crucial role in various biological processes including the regulation of normal physiological processes (Morrow et al., 2010; Nathan, 2003), however in excess, can lead to an imbalance in the redox environment and lead to a variety of disorders including cancer, I/R injury, and neurodegeneration (Dirani et al., 2011; Hess and Manson, 1984; Lin and Beal, 2006; Liu et al., 2007; Morrow et al., 2010; Tezel, 2006; Yapici et al., 2011). Techniques capable of quantifying and visualising the changes to the cellular redox environment of biological systems as a result of pro-oxidant or antioxidant processes are therefore crucial for understanding the mechanistic links between free radical chemistry and disease outcomes (Morrow et al., 2010). The most effective way to define this link is with the real-time measurement of redox changes occurring in live cells. We have designed and synthesized a novel reversible profluorescent probe, ME-TRN, based on a rhodamine fluorophore containing a nitroxide functional group. Here we demonstrate the successful application and *in vivo* evaluation of ME-TRN for the detection and quantification of retinal oxidative status in real-time.

The retina is an ideal model for examining ROS-mediated pathological events, due to the high content of polysaturated fatty acid and high oxygen consumption (Bazan, 1988; Li et al.,

2009; Li and Lo, 2010). The rat retinal I/R model, which mimics clinical situations such as retinal vascular occlusion disease and acute glaucoma, is an established animal model for studying retinal cell responses after an ischemic insult (Cho et al., 2011; Sun et al., 2010). It is believed that impairment of mitochondrial integrity is a key factor in ROS-mediated neurodegeneration (Chrysostomou et al., 2013; Osborne and del Olmo-Aguado, 2013). Cellular events such as disruption of ion homeostasis, depletion of adenosine triphosphate stores and glutamate-induced excitotoxicity (Osborne et al., 2004; Pellegrini-Giampietro et al., 1990), occur during the challenge of oxidative stress (Aydemir et al., 2004; Block and Schwarz, 1997; Celebi et al., 2001; Li and Lo, 2010) resulting in increased levels of lipid peroxidation, depletion of free radical scavengers (Block and Schwarz, 1997; Chidlow et al., 2002) and subsequent neurodegeneration. Conversely, the reduction of free radical formation and reduced oxidative stress can retard or prevent neuronal cell death in ischemic retina (Chidlow et al., 2002; Dilsiz et al., 2006; Li et al., 2009; Maher and Hanneken, 2008). ROS detection methods and antioxidants that target the mitochondria are therefore of high interest and importance.

We have previously shown through confocal microscopy, that ME-TRN is taken up by living cells (RGC-5 cell line and fibroblasts), and is selectively accumulated in the mitochondria (Barnett et al., 2013). Similar nitroxide hybrids have been shown to localize to the mitochondria (Smith et al., 2008). The unique potential of our nitroxide-based probe to interconvert reversibly between the stable nitroxyl radical, the reduced hydroxylamine and oxidized oxoammonium forms has allowed for the first time, detection of ROS and the possible benefits of antioxidant therapy to be monitored *in vivo*, in real time.

The ME-TRN probe response is driven by the overall reducing processes of the cellular environment and, in particular, by the mitochondria. The presence of the stable radical during oxidation short-circuits the normal fluorescence effect and so these systems possess low inherent fluorescent emission. Upon metabolism, redox processes or free radical scavenging reactions, the free radical is converted to the non-radical species that displays the bright fluorescence of the inherent chromophore (Blough and Simpson, 1988; Morrow et al., 2010). Previously, this redox-response of the probe was observed i*n vitro* by stimulating superoxide production in fibroblast and RGC-5 cell cultures, through the inhibition of the mitochondrial respiratory chain with antimycin (Barnett et al., 2013). Here, for the first time, we show a similar response *in vivo* (rat retina I/R injury), demonstrating a significant decrease in probe

fluorescence in response to a pro-oxidant stress. This fluorescence decrease was time dependent ($F_{(5,30)} = 2.864$, $p = 0.031$, see Fig. 5), signifying the increased production and accumulation of ROS within the cellular environment during the reperfusion phase. The reduction of free radicals and oxidative stress through the administration of lutein (Dilsiz et al., 2006; Li et al., 2009) successfully ameliorated the I/R-induced decrease in retinal fluorescence. Antioxidant therapy can therefore be considered somewhat effective at maintaining the probe in the fluorescent reduced hydroxylamine state, limiting oxidation back to the nitroxyl radical form.

During the past decade, numerous studies have investigated neuroprotective strategies to reduce / prevent retinal cell death. Recently, intensive efforts have been made to explicate the neuroprotective effects of carotenoids in ocular diseases *in vivo* (Li et al., 2009; Muriach et al., 2006; Sasaki et al., 2009) and *in vitro* (Li and Lo, 2010; Nakajima et al., 2009). Various carotenoids are present in human plasma, but only the xanthophylls lutein and zeaxanthin are found in retina in considerable amounts (Junghans et al., 2001). Lutein, a potent antioxidant, has been applied in human clinical trials and shown to improve vision and retard the progression of AMD and cataract development (Chasan-Taber et al., 1999; Itagaki et al., 2006; Li et al., 2009; Li and Lo, 2010; Richer et al., 2007; Richer et al., 2004), however the mechanism of protection is unclear and the role of lutein in ischemic injury is limited (Li et al., 2012). *In vitro* studies show lutein can penetrate into cells and scavenge intracellular H_2O_2 preventing cell damage (Li and Lo, 2010) suggesting a possible therapeutic benefit to antioxidant intervention for diseases linked to oxidative stress. Our present results confirm that a single administration of lutein provides a degree of protection against oxidative stress. Whether a prolonged diet rich in antioxidants prior to a future ischemic injury could mitigate the deleterious effects of ROS requires further investigation.

This study highlights the ability of the novel ME-TRN probe to detect, visualize and quantify oxidative stress *in vivo*. ERG analysis of a- and b-waves and OPs, also demonstrated that intraocular injection of ME-TRN had no effect on the functional integrity of the retina (Fig. 2). Moreover, normal retinal status was confirmed in ME-TRN treated retinas through immunohistochemical studies: GFAP expression remained confined to astrocytes and IBA-1 labelled microglia demonstrated minimal activation. This was in stark contrast to the glial cell activation we and others see in injured retinas (Fig. 3) (Larsen and Osborne, 1996; Naskar et al., 2002; Wang et al., 2000). These data suggest that ME-TRN can be safely administered in

the eye to monitor the dynamic processes of oxidative stress. In this study, we used ME-TRN to detect ROS production in an acute model of ischaemia, which predominantly induces inner retinal damage. Figure 1 demonstrates that ME-TRN is not only taken up by the inner retinal neurons, but is also avidly accumulated in the outer nuclear layer. The advantage of ME-TRN accumulation throughout the retina should also allow the detection of ROS production in models of outer retinal disease such as age-related macular degeneration and retinal dystrophies.

5. Conclusion

In summary, this study documents a new technique for the real-time measurement of retinal oxidative status *in vivo*. This could potentially have a major impact on the ability to assess putative neuroprotective antioxidants for the treatment of retinal and other neurodegenerative diseases. The mechanisms underlying retinal degeneration in a number of ocular disease states like glaucoma, age-related macular degeneration and retinal ischemia are complex. However it is becoming increasingly evident that mitochondrial dysfunction, superoxide generation and oxidative stress play a significant role. The ability to quantify such changes *in vivo* is of great value. Reversible PFN probes provide a possible avenue for this quantification. The ME-TRN probe has proven effective at detecting alterations in the cellular redox status *in vivo* and hence provides a unique investigative tool allowing real-time insight into a variety of disorders linked to oxidative stress.

Conflicts of Interest

The authors report no conflict of interest.

Author contribution

The authors alone are responsible for the content and writing of this paper.

CLR conducted much of the experimental work including the induction of ischemia/reperfusion, fluorescence imaging, immunohistochemistry and electroretinography, and wrote the first draft of the manuscript.

GAG conducted and provided guidance for intraocular injections and fundus imaging. He provided significant intellectual input into the final manuscript.

SEB, as the developer of the ME-TRN probe, was involved in the conception of the project and all intellectual aspects of the nitroxide chemistry. He provided significant input into the final manuscript.

NLB conceived and designed the project, conducted all initial experiments, analyzed the data and provided significant input into the final manuscript.

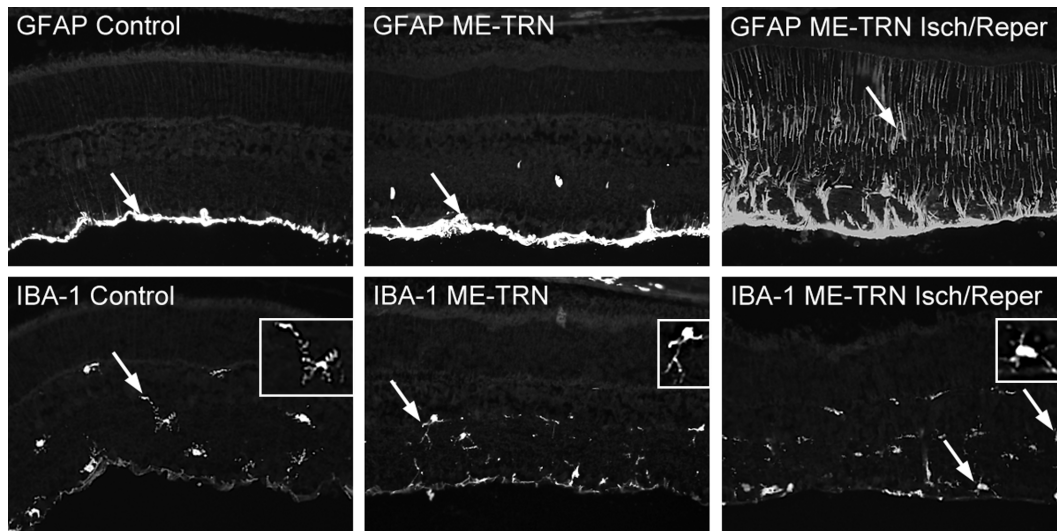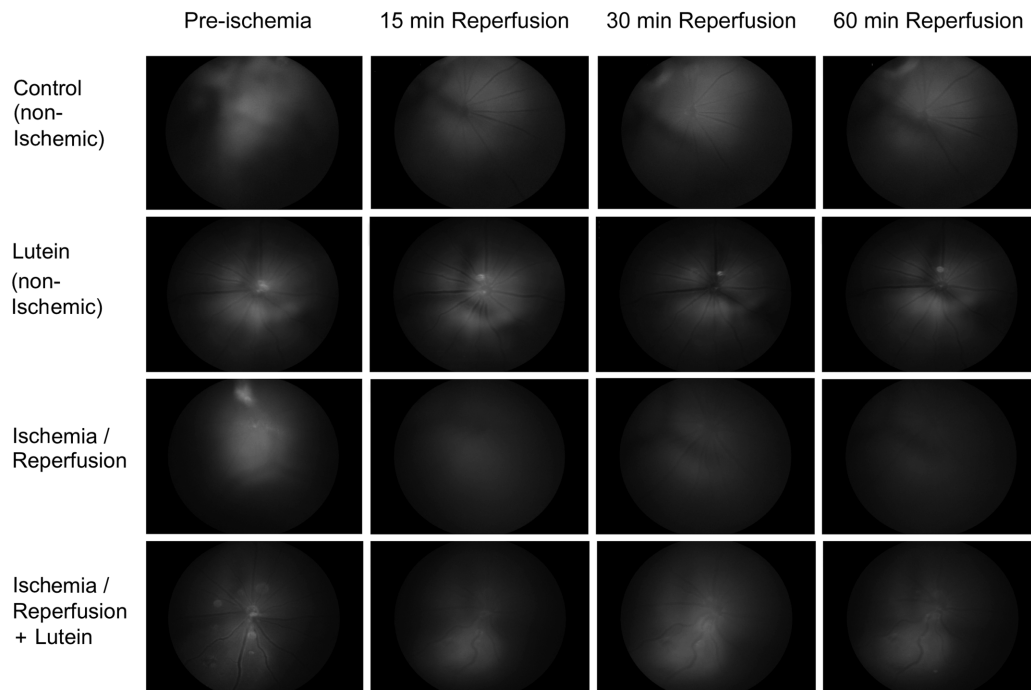**FIGURES**
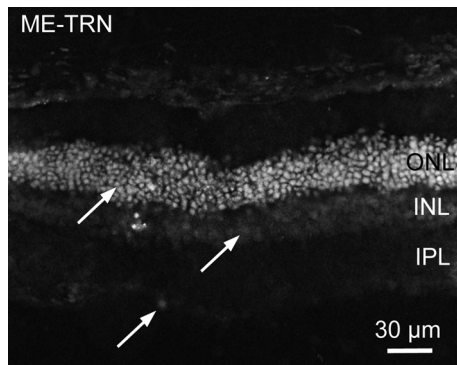
FIGURE 1: Transverse frozen section of rat retina showing accumulation and fluorescence of ME-TRN probe in neurons (arrows) 60 mins after a single intravitreal injection (2 µM). Brightest fluorescence seen in the outer nuclear layer (ONL) with weaker fluorescence displayed by cells of the inner nuclear layer (INL) and ganglion cells; IPL, inner plexiform layer.
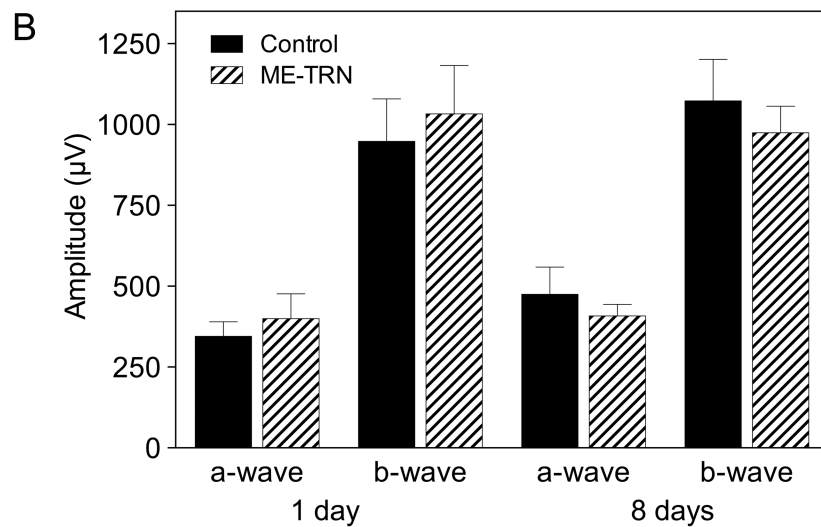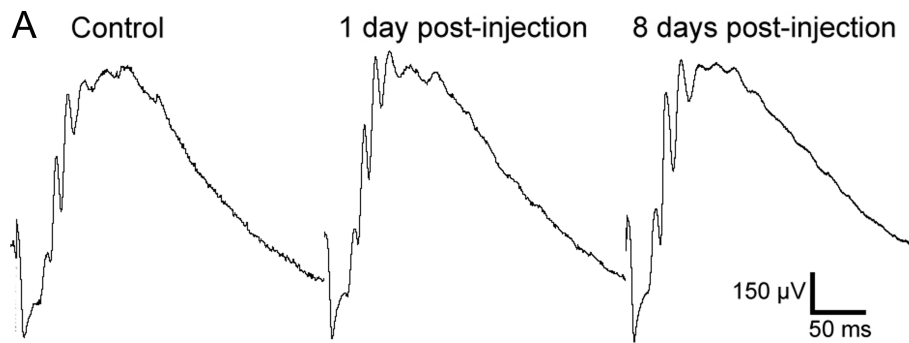
FIGURE 2: ERG analysis of vehicle control and ME-TRN injected eyes, 1 and 8 days post treatment (n= 3, mean ± SEM). (a) Representative traces of the scotopic ERG waveforms recorded with a stimulus of 1.2 log cd.s.m^{-2} showing no apparent change in a-wave, b-wave or oscillatory potential amplitude or peak latency. (b) Quantification of a- and b-wave amplitudes confirming no significant effect of ME-TRN (2 µM) injection upon retinal function.

FIGURE 3: GFAP and IBA-1 immunohistochemistry of rat retinas 8 days after the intravitreal injection of ME-TRN (2 μM). No differences in labelling were apparent between control and ME-TRN trerated retinas. GFAP immunoreactivity was restricted to the astrocytes whilst IBA-1 positive microglia displayed the resting ramified morphology. In contrast, an ischemic insult followed by 8 days of reperfusion resulted in a significant upregulation in GFAP immunoreactivity associated with activated Müller cells. I/R also resulted in the activation of microglia (IBA-1), which displayed a rounded nucleus, abundant cytoplasm and shorter processes. Higher magnification insets show microglia nuclei in each condition of activation.

FIGURE 4: *In vivo* fundus imaging showing the time-course of ME-TRN fluorescence (556/590 nm) in the rat eye during reperfusion following an acute ischemic insult, and the effect of the antioxidant lutein (0.2 mg/kg). Fundus fluorescence was stable in non-ischemic retinas throughout the 60 min 'reperfusion' period (top rows). In contrast, reactive oxygen species generated during the reperfusion phase after acute ischemia resulted in a marked, time-dependent, decrease in probe fluorescence (third row). Pre-ischemic antioxidant treatment ameliorated the I/R-induced decrease in retinal fluorescence (bottom row).
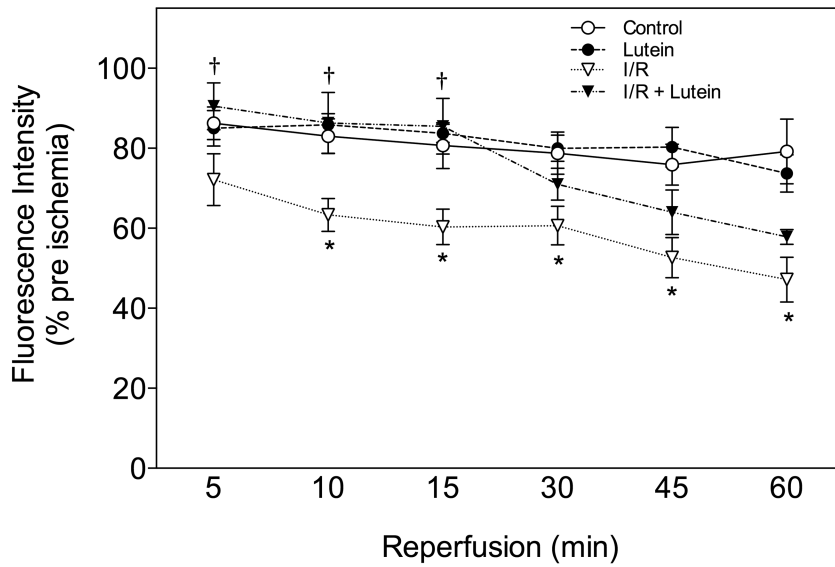
FIGURE 5: Quantification of ME-TRN fluorescence captured in the rat fundus *in* vivo during 60 mins of reperfusion following an acute ischemic insult. Data presented as the change in fluorescence intensity at each time point as a percentage of the pre-ischemic (time = 0) value for each eye (mean ± SEM). An ischemic insult resulted in a marked decrease in fluorescent intensity over the 60 mins of reperfusion (I/R) when compared to the non-ischemic control group, with significant reductions seen at 10, 15, 30, 45 and 60 mins reperfusion (* $p < 0.05$ compared with non-ischemic control). An intraperitoneal injection of lutein (0.2 mg/kg) 1 hour prior to the ischemic insult ameliorated the decrease in fluorescence observed in I/R animals, with significant improvements observed after 5, 10 and 15 mins of reperfusion († $p < 0.05$ compared with I/R alone).

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