Experimental Eye Research 169 (2018) 1–12

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

670nm light treatment following retinal injury modulates Müller cell gliosis: Evidence from *in vivo* and *in vitro* stress models

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ARTICLE INFO

Keywords:
Müller cell
Gliosis
Retinal degeneration
670 nm light
Inflammation

ABSTRACT

Photobiomodulation (PBM) with 670 nm light has been shown to accelerate wound healing in soft tissue injuries, and also to protect neuronal tissues. However, little data exist on its effects on the non-neuronal components of the retina, such as Müller cells (MCs), which are the principal macroglia of the retina that play a role in maintaining retinal homeostasis. The aim of this study was to explore the effects of 670 nm light on activated MCs using *in vivo* and *in vitro* stress models. Adult Sprague-Dawley rats were exposed to photo-oxidative damage (PD) for 24 h and treated with 670 nm light at 0, 3 and 14 days after PD. Tissue was collected at 30 days post-PD for analysis. Using the *in vitro* scratch model with a human MC line (MIO-M1), area coverage and cellular stress were analysed following treatment with 670 nm light. We showed that early treatment with 670 nm light after PD reduced MC activation, lowering the retinal expression of GFAP and FGF-2. 670 nm light treatment mitigated the production of MC-related pro-inflammatory cytokines (including IL-1β), and reduced microglia/macrophage (MG/MΦ) recruitment into the outer retina following PD. This subsequently decreased photoreceptor loss, slowing the progression of retinal degeneration. *In vitro*, we showed that 670 nm light directly modulated MC activation, reducing rates of area coverage by suppressing cellular proliferation and spreading. This study indicates that 670 nm light treatment post-injury may have therapeutic benefit when administered shortly after retinal damage, and could be useful for retinal degenerations where MC gliosis is a feature of disease progression.

1. Introduction

Müller cells (MCs) are the principal macroglia of the vertebrate retina and play a key role in maintaining retinal structure and homeostasis. During retinal injury, the early response of MCs involves the release of neuroprotective factors such as ciliary neurotrophic factor (CNTF), and fibroblast growth factor (FGF-2) (Bringmann and Wiedemann, 2012; Shen et al., 2012), which have been shown to rescue photoreceptors following photo-oxidative damage (Valter et al., 2005). Activated MCs express chemokine C-C motif ligand 2 (CCL2), a known chemoattractant and activator for MG/MΦ *in vitro* (Matsushima et al., 1989; Nakazawa et al., 2007a; Yoshimura et al., 1989). Exposure to PD *in vivo* induces retinal *Ccl2* expression in MCs, and subsequent recruitment of MG/MΦ to areas of severe damage (Rutar et al., 2012b). In severe retinal damage, when a large number of neurons are lost, MCs enter into proliferative gliosis forming glial scars (Bringmann et al., 2009), which create barriers that hinder nutrient delivery to surviving retinal neurons, causing further cell death leading to disease progression (Albarracin and Valter, 2012).

PBM is low-energy photo-irradiation that has been shown to accelerate wound healing in skin (Conlan et al., 1996), mucosa (Desmet et al., 2006), and soft tissue (Herranz-Aparicio et al., 2013). Beneficial effects have also been reported in central nervous system (CNS) tissue injuries involving the spinal cord, retina and optic nerve (Lawrence et al., 2007). Pre-clinical studies in models of retinal degeneration have demonstrated that 670 nm light treatment is neuroprotective, can slow photoreceptor and ganglion cell death (Albarracin et al., 2011, 2013; Eells et al., 2003; Giacci et al., 2014; Natoli et al., 2013; Tang et al., 2013), and reduce pro-inflammatory cytokine secretion, macrophage recruitment and complement activation (Calaza et al., 2015; Kokkinopoulos, 2013; Kokkinopoulos et al., 2013). Acknowledging these benefits, it has recently gained FDA approval for clinical use (Desmet et al., 2006; Fitzgerald et al., 2013; Whelan et al., 2001). Clinical studies over the past decade have further demonstrated the

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https://doi.org/10.1016/j.exer.2018.01.011
Received 14 November 2017; Received in revised form 4 January 2018; Accepted 12 January 2018

neuroprotective effect of 670 nm light in retinal degenerations (Ivandic and Ivandic, 2008, 2012; Merry et al., 2017; Tang et al., 2014).

Although the effects of 670 nm light on retinal neurons have been documented, the influence of 670 nm light on non-neuronal cells, including MCs, has not been well-studied. We previously demonstrated that 670 nm light treatment prior to retinal injury could ameliorate the activation of MCs following photo-oxidative damage (Albarracin et al., 2011; Albarracin and Valter, 2012). However, the effects of administering 670 nm light treatment after photo-oxidative damage have not been fully investigated. In this study, we explored the direct effect of 670 nm light on activated MCs using *in vivo* and *in vitro* models of retinal stress.

2. Materials and methods

2.1. Animals and PD

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the Australian National University Animal Experimentation Ethics Committee (A2014/56). Thirty-nine adult albino Sprague-Dawley (SD) rats aged 100–120 postnatal days (P) were used for *in vivo* experiments. Animals were born and raised in low light levels (5 lux) in a 12-h light, 12-h dark cycle with food and water available *ad libitum*. PD was performed as described previously (Albarracin and Valter, 2012). Briefly, animals were exposed to white light (1000 lux) for 24 h using transparent Perspex open-top cages placed under a light source (COLDF2, 2 × 36W, IHF; Thorn Lighting, Spennymoor, UK). Animals were returned to a low light environment (5 lux) to recover for 30 days; some animals were treated with 670 nm light during recovery. Animals were euthanized and tissue was collected at 30 days after PD.

2.2. PBM with 670 nm light in vivo

Animals were divided into 5 groups: **PD + R0** (PBM commenced immediately after PD, n = 7); **PD + R3** (PBM commenced at 3 days after PD, n = 10); **PD + R14** (PBM commenced at 14 days after PD, n = 7); **PD** (animals were exposed to PD only, n = 9); and **control** (animals were not exposed to PD or 670 nm light, n = 6). In the treatment groups (PD + R0, PD + R3, PD + R14) PBM was performed using a 670 nm light-emitting diode array (WARP 75; Quantum Devices, WI, USA) during the period of recovery, commencing at the specified times post-PD. During treatment, animals were positioned so that both eyes were approximately 2.5 cm away from the light source and were exposed to 670 nm light for 3 min at 60 mW/cm^2, delivering 9J/cm^2 irradiation to the retina, daily for 5 consecutive days.

2.3. Uniform scratch model using MIO-M1 cells

A spontaneously immortalized human Müller cell line, MIO-M1 (Limb et al., 2002b) was validated for species authenticity (CellBank, Sydney, Australia) and used for *in vitro* experiments. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), supplemented with 6 mM L-glutamine (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded on 6-well plates (1.3×10^5 per well), on 8-well chamber slides (1.3×10^4 per well) (Millipore, MA, USA), or on transwell inserts (5 μm pore-size; Millipore) (3000/insert) for 2 day at 37 °C in a humidified CO_2 incubator to reach 90% confluence. A uniform scratch was created in each well using a 1 ml pipette tip, and a grid was used beneath each well to ensure uniformity between wells. Floating cells and debris were removed by rinsing cells with 0.1M PBS, before wells were replenished with fresh culture growth medium.

To inhibit proliferation, some cells (6-well plates) were incubated with Mitomycin C (MMC, 10 μg/ml, Sigma Aldrich) for 2 h, washed with 0.1M PBS (Arranz-Valsero et al., 2014), and growth medium was replenished prior to the scratch injury.

2.4. 670 nm light treatment in vitro

For PBM of cells, a 670 nm LED array (WARP 10; Quantum Devices) was used. Cells were irradiated 3 times with 670 nm light for 3 min at 60 mW/cm^2 (9J/cm^2). The first PBM treatment was delivered immediately after the scratch, followed by two further irradiations at 4-h intervals in the first 24 h. Four experimental groups were used: **non-scratch (NS)** control, where cells did not undergo scratch injury or 670 nm treatment; **scratch (S)**, where cells received scratch injury and sham 670 nm treatment (cells underwent identical handling to the 670 nm treatment groups, without the light irradiation); **NS + 670** and **S + 670** groups both received 670 nm treatment (N = 3 wells/group, N = 3 repeats). Baseline measurements were taken immediately following the first intervention for each group (scratch ± 670 nm) – this was designated T = 0. Measurements were performed at T = 24, 48, and 72 h. Digital images of scratch edges were captured using a phase-contrast light microscope (Axiovert 3; Zeiss, Oberkochen, Germany). The progression of cell coverage across the scratch area was analysed by measuring the area covered (pixels) from the edge of the original scratch (T = 0), using ImageJ software (National Institutes of Health, MA, USA).

2.5. Cell migration assay

Cells were seeded on transwell inserts (3000 cells/insert) for analysing cell migration. To initiate migration, serum-free medium was added to the cells on the inserts, while growth medium was added to the lower chamber. After 24, 48 or 72 h, cells on the inserts were fixed with 4% paraformaldehyde for 10 min at room temperature, and stained with bisbenzimide (BBZ, 1:10000; Sigma-Aldrich). The non-migrating cells on the surface of the insert membrane were removed using cotton swabs. To measure cell migration across the insert membrane, the number of cells on the lower surface were visualised and counted using a laser-scanning A1$^+$ confocal microscope (Nikon, Tokyo, Japan) (Lu et al., 2013).

2.6. Flow cytometry analysis for cell viability and cell cycle

Flow cytometry was used to assess cell cycle status and cell death. Vybrant DyeCycle Violet Stain (Thermo Fisher Scientific) was utilized to recognize live cells, and 7-aminoactinomycin D (7-AAD, Thermo Fisher Scientific) was used to identify dead cells. Briefly, cells were trypsinized and collected from 6-well plates. Cells were incubated with 1 μl of Vybrant DyeCycle Violet Stain diluted in 1 ml Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific) for 2 h at 37 °C in the dark, and then was rinsed twice with 0.1M PBS. Cell pellets were re-suspended in HBSS containing 2.5% 7-AAD and were incubated for 10 min at 37 °C in the dark. Fluorescence was detected using flow cytometry (LSRII; BD, CA, USA) and analysed using FlowJo (FlowJo, OR, USA).

2.7. Immunofluorescent staining on retinal tissues and cells

Following 30 days recovery, animals were euthanized using an intraperitoneal injection of barbiturate (Valabarb; Virbac, NSW, Australia). One eye of each animal was enucleated, immersion fixed in 4% paraformaldehyde and then cryoprotected with 15% sucrose overnight. Eyes were cryosectioned at 16 μm thickness. Immunofluorescent staining was performed as previously described (Albarracin et al., 2011; Albarracin and Valter, 2012), using primary antibodies listed in Table 1. Following incubation with a fluorophore-conjugated secondary antibody, the cell nuclei were stained with bisbenzimide. To quantify