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Topical Coenzyme Q10 demonstrates mitochondrial-mediated neuroprotection in a rodent model of ocular hypertension

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ABSTRACT

Coenzyme Q10 (CoQ10) is a mitochondrial-targeted antioxidant with known neuroprotective activity. Its ocular effects when co-solubilised with α–tocopherol polyethylene glycol succinate (TPGS) were evaluated. *In vitro* studies confirmed that CoQ10 was significantly protective in different retinal ganglion cell (RGC) models. *In vivo* studies in Adult Dark Agouti (DA) rats with unilateral surgically-induced ocular hypertension (OHT) treated with either CoQ10/TPGS micelles or TPGS vehicle twice daily for three weeks were performed, following which retinal cell health was assessed *in vivo* using DARC (Detection of Apoptotic Retinal Cells) and post-mortem with Brn3a histological assessment on whole retinal mounts. CoQ10/TPGS showed a significant neuroprotective effect compared to control with DARC ($p < 0.05$) and Brn3 ($p < 0.01$). Topical CoQ10 appears an effective therapy preventing RGC apoptosis and loss in glaucoma-related models.

1. Introduction

Glaucoma is a progressive neurodegenerative eye disorder estimated to affect 60 million people worldwide (Cook and Foster, 2012; Tham et al., 2014). Glaucoma involves the progressive loss of retinal ganglion cells (RGCs) and their axons, which results in visual field abnormalities and ultimately blindness if left untreated (Garcia-Valenzuela et al., 1995; Quigley et al., 1995). Elevated intraocular pressure (IOP) is presently the only modifiable disease risk factor (Weinreb and Khaw, 2004; Lee et al., 2014a). However, recognition of a subset of glaucoma patients who continue to exhibit visual decline despite therapeutically well-controlled IOP has led to the realisation that novel therapeutic paradigms for this condition are urgently required (Resnikoff et al., 2004).

RGC loss in glaucoma is predominantly thought to occur *via* elevated apoptosis (a type of programmed cell death) (Quigley et al., 1995; Cordeiro et al., 2010) which is mainly mitochondrial dysfunction mediated (Lee et al., 2014a; Ju et al., 2008; Park et al., 2011). While the primary site of injury is thought to occur at the site of the RGC axon in the optic nerve, (Quigley et al., 1977; Minckler et al., 1977; Quigley et al., 1981; Knox et al., 2007) the resulting loss of RGCs (primary degeneration) can also lead to the secretion of pro-apoptotic factors resulting in secondary neurodegeneration and the death of neighbouring RGCs (Davis et al., 2016a). Although the exact mechanism of glaucoma progression remains to be elucidated, elevated oxidative stress has been suggested to contribute to glaucoma pathogenesis (Tezel et al., 2005; Yuki et al., 2010). Mitochondria are a source and target of oxidative stress and therefore are key in the development of neuroprotective strategies for RGC preservation in glaucoma (Chrysostomou et al., 2013).

Coenzyme Q10 (CoQ10) is a mitochondrial targeted antioxidant that plays an essential role in the normal function of the electron transport chain. CoQ10 has been reported to exhibit neuroprotective activity in a range of disorders including; cerebral ischemia, (Ahmed et al., 2015) Parkinson's disease and Huntington's disease (Klongpanichapak et al., 2006). In addition to its role as an antioxidant, CoQ10 is also reported to protect against glutamate excitotoxicity *in vivo* through the inhibition of mitochondrial depolarization (Papucci et al., 2003; Lee et al., 2014b).

Concentrations of CoQ10 in the human retina are reported to decline by up to 40% with age (Qu et al., 2009). The poor aqueous solubility (Fato et al., 2010) and low bioavailability of CoQ10, due in part to its interactions with the multi-drug efflux pump P-glycoprotein (P-gp), have limited the development of topically active formulations of

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this drug (Hirano and Iseki, 2008). The interaction of CoQ10 with P-gp, expressed in both corneal epithelial cells (Vellonen et al., 2010) and RGCs (Duncan et al., n.d.) suggests that co-administration of CoQ10 with a P-gp inhibitor would likely enhance the topical delivery and pharmacological effects of this drug (Hirano and Iseki, 2008). α-Tocopherol is a form of vitamin E best known for its role as a lipid soluble antioxidant but is well-documented to inhibit P-glycoprotein (P-gp) activity (Wu et al., 2007; Davis et al., 2015). The mechanism of α-Tocopherol mediated P-gp inhibition is poorly understood but has recently been suggested to occur as a result of indirect modulation of the membrane dipole potential (Davis et al., 2015).

Formulation of CoQ10 into micelles using the vitamin E derivative D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) has previously been reported to deliver micromolar concentrations of CoQ10 to the vitreous in patients 1 h after administration (Fato et al., 2010). The present study sought to investigate the mechanism of α-Tocopherol mediated P-gp inhibition and assess the neuroprotective effects of CoQ10 and TPGS using immortalised and primary mixed retinal cultures (Galvao et al., 2014; McCarthy et al., 2004). Finally, the efficacy of topically applied CoQ10/TPGS micelles was next evaluated *in vivo* using the well-established Morrison's ocular hypertension model (OHT) (Morrison et al., 1997) and *in vivo* DARC (Cordeiro et al., 2017) and Brn3a-RGC immunohistochemistry as endpoints (Galvao et al., 2013; Davis et al., 2016b).

2. Methods

2.1. Cell culture

Both primary murine retinal mixed cultures (pMC) and an immortalised retinal neuronal (RN) cell line (RGC5, a gift from Dr. Neeraj Agarwal, Department of Cell Biology and Genetics, UNT Health Science Centre, Fort Worth, TX) were used. These cells express retinal neuronal proteins Thy-1, Brn3a, and β3 tubulin (Krishnamoorthy et al., 2001; Burugula et al., 2011; Nadal-Nicolás et al., 2009), and are known to be similar to the 661w photoreceptor cell line and RGCs (Al-Ubaidi, 2014; Van Bergen et al., 2009; Krishnamoorthy et al., 2013). RN were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin. Primary murine (C57BL/6) mixed retinal cultures were isolated from P1 pups and neuronal cells isolated by incubation in a solution containing 10 units of papain/mL, and cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen, UK), 100 U/mL penicillin, 100 μg/mL of streptomycin and 0.292 mg/mL glutamine (Gibco, UK), 7.5% sterile dH20 and 1.5 mM KCl (Sigma-Aldrich, UK). The medium was changed completely on day 1 and 50% refreshed on day 2. Cells were used for experiments on day 3.

2.2. P-glycoprotein activity assessment

Analysis of P-gp activity was performed as previously described (Ohashi et al., 2006). Briefly, RN cells were seeded at 4000 cells/well in a 96 well plate for 24 h. On the day of the study, cell monolayers were washed before treatment with varying concentrations of TPGS or verapamil hydrochloride (Sigma-Aldrich), a known P-gp inhibitor for 10 min and incubated for 10 min at 37 °C. After this time, cells were incubated with the P-gp substrate calcein-AM (Invitrogen) for 60 min before P-gp activity was measured by quantifying calcein fluorescence using excitation and emission wavelengths of 485 nm and 530 nm respectively (Safire plate reader). Percentage P-gp activity at each concentration of drug was determined using Eq. (1);

$$\text{Pgp activity } (\%) = 100 - \frac{(RFU_{test} - RFU_{BK})}{(RFU_{MAX} - RFU_{BK})} \quad (1)$$

where; RFU_{test} is the fluorescence in the presence of test compound, RFU_{BK} is the fluorescence in the absence of test compounds and RFU_{MAX} is the fluorescence in the presence of 66 μM verapamil which induced maximal P-gp inhibition. EC_{50} values were determined by fitting results to four-parameter dose response curves.

2.3. Dipole potential assessment

RN cultures were seeded at 4000 cells/well in a 96 well plate and permitted to settle for 24 h before washing well before labelling with 0.5 μM of the fluorescent probe di-8-ANEPPs (Invitrogen, from 2 mM stock solution in ethanol) for 1.5 h in phenol-red free DMEM (Sigma-Aldrich) (Davis et al., 2015). After this time the ratiometric di-8-ANEPS fluorescence intensity at excitation of 420/520 nm and emission of 670 nm using a Safire plate reader for each cell population was recorded before and 10 min after cells were treated with varying concentrations of TPGS for 10 min. The change in fluorescence ratio of di-8-ANEPPS indicates a change in the membrane dipole potential on addition of an agent of interest. The dissociation constant (K_d) of the interaction of TPGS for neuronal cells was determined by fitting the change in di-8-ANEPPs fluorescence ratio to a hyperbolic binding equation as described previously (Davis et al., 2010).

2.4. Immunocytochemistry

pMC were fixed in 4% paraformaldehyde for 15 min before washing twice with PBS and permeabilizing in PBS plus 0.1% Tween-20. Cells were blocked with PBS containing 3% bovine serum albumin (BSA, Sigma-Aldrich, UK) for 1 h prior to incubation with primary antibodies overnight at 4 °C (diluted in PBS containing 3% BSA; see Table 1 for details of antibodies used), followed by the appropriate Alexa Fluor 488 nm or 555 nm secondary antibody for a further hour at a 1:1000 dilution (Life technology, UK). Cells were subsequently washed twice with PBS, before addition of 5 μg/mL cell permeable dye Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 5 min at room temperature prior to visualisation. Then mounted with mowiol (Merck, UK) and were observed under a confocal fluorescence microscope (LSM 700, Carl Zeiss MicroImaging GmbH, Jena, Germany).

2.5. Reverse transcription PCR assay

To test pMC for retinal neuronal marker expression, total RNA was extracted from primary mixed retinal cultures using RNeasy mini kit following manufacturer's specifications (Qiagen, UK). Complementary DNA (cDNA) synthesis was conducted by QuantiTect Reverse Transcription (Qiagen) according to manufacturer's protocol. The PCR reaction was conducted using the GoTaq G2 DNA polymerase kit (Promega, UK). Primers and cycle conditions are summarised Table 2.

2.6. Oxidative cytotoxicity evaluation and cell viability assays

pMC were plated at 30,000 cell/well in 96-well plates for 24 h. After this time cells were treated with either 20 μM CoQ10 with 57 μM TPGS, or 57 μM TPGS only (vehicle control) for 2 h. The molar ratio of CoQ10 and TPGS chosen was the same as that present in the micelle formulation subsequently used *in vivo*. After this time, treatments were removed before application of varying concentrations of cytotoxic

Table 1
Antibodies source and optimized dilutions.

Antibody	Company	Cat.	Host species	Dilution
Brn3a	Abcam	AB81213	Rabbit	1:200
Thy-1	Abcam	AB225	Mouse	1:500
RBPMS	Abcam	AB152101	Rabbit	1:500
γ-synuclein	Abcam	AB55424	Rabbit	1:1200

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