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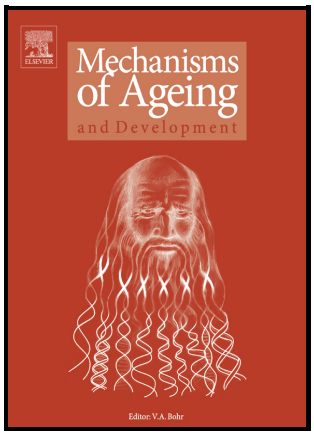

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Deuterated polyunsaturated fatty acids provided protection against oxidative stress in ocular fibroblasts derived from glaucoma patients.

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Abstract

Glaucoma is a complex neurodegenerative disease of the optic nerve that leads to irreversible sight loss. Lowering intraocular pressure (IOP) medically or surgically represents the mainstay of treatment but despite adequate treatment optic nerve function can continue to deteriorate leading to blindness. There is significant clinical and experimental evidence that oxidative stress is involved in the pathogenesis of glaucoma. Decreasing the formation of lipid peroxidation products or scavenging them chemically could be beneficial in limiting the deleterious effects of oxidative stress in glaucoma. A solution to control the susceptibility of PUFAs to noxious lipid peroxidation reactions is by regioselective deuteration. Deuterium incorporated into PUFAs at bis-allylic positions (D-PUFAs) inhibits the rate-limiting step of lipid peroxidation. In this study, we have shown that Tenon's ocular fibroblasts from glaucoma patients have significantly increased basal oxidative stress compared to non-glaucomatous control patients. Furthermore, we have shown that deuterated polyunsaturated fatty acids (D-PUFAs) provide an enhanced rescue of menadione induced lipid peroxidation in both non-glaucomatous and glaucomatous Tenon's ocular fibroblasts using malondialdehyde (MDA) levels as a marker. Our study suggests that D- PUFAs may provide a potentially safe and effective method to reduce cytotoxic oxidative stress in glaucoma.

Abbreviations

RGC: retinal ganglion cell, POAG: primary open angle glaucoma, XFG: pseudo-exfoliation glaucoma, IOP: intraocular pressure, PUFA: polyunsaturated fatty acid, H-PUFAs: nondeuterated PUFAs, LIN: linoleic acid, LNN: linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, D-PUFAs: deuterated poly-unsaturated fatty acids, D2-LIN: D2-linoleic acid, D4-LNN: D4-linolenic acid, D8-EPA: D8-eicosapentaenoic acid, and D10-DHA: D10-docosahexaenoic acid, ROS: reactive oxygen species, TFs: Tenon's ocular fibroblasts, GTF: glaucomatous Tenon's ocular fibroblasts, NTF: non-glaucomatous Tenon's ocular fibroblasts, RFU: relative fluorescence units, MDA: malondialdehyde.

1. Introduction

Glaucoma is the leading cause of irreversible blindness worldwide affecting over 60 million people; a number which is predicted to 118.8 million by 2040[1]. The pathogenesis of glaucoma is complex but ultimately there is retinal ganglion cell (RGC) death and characteristic optic nerve head cupping and resultant visual field defects[2,3]. Primary open angle glaucoma (POAG) is the commonest subtype of glaucoma[4] and pseudo-exfoliation glaucoma (XFG) is the most prevalent type of secondary open angle glaucoma[5]. There is overwhelming evidence from several prospective randomised multi-centre studies showing that the reduction of intra-ocular pressure (IOP) is neuro-protective, in the sense that it delays or even prevents the structural and functional damage of optic nerve axons in glaucoma[6]. However, even when IOP is adequately controlled between 12-45% of POAG patients continue to show progressive visual field loss[7,8]. Despite appropriate management of glaucoma by IOP reduction, the cumulative risk of unilateral blindness due to glaucoma is 26.5% after 10 years and 38.1% at 20 years; and bilateral blindness is 5.5% at 10 years and 13.5% at 20 years[9]. There is therefore a compelling need to develop IOP-independent neuroprotective therapies[10,11].

Oxidative stress is induced through the formation of multiple reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals that can initiate and propagate free radicals. The net oxidative burden between the prooxidant and antioxidant systems is oxidative stress, which damages cellular and tissue macromolecules such as lipids, proteins, and nucleic acids, and results in cellular and tissue dysfunction and subsequent cellular death. The systemic status of redox in glaucoma has been the subject of increasing interest following identification of the circulating autoantibodies against antioxidative stress enzymes in the serum of patients with glaucoma[12]. Several studies have reported on this topic and a meta-analysis confirmed elevated serum levels of oxidative stress markers in glaucoma[13]. Sequential studies from Japan have reported evaluated systemic oxidative stress in both POAG and XFG using global measures of oxidant and anti-oxidant status[14–17]. Specifically, there was a reduction in systemic antioxidant capacity level in POAG and

XFG subjects and this was correlated with elevated IOP[17] and greater visual field loss[16]. There is significant experimental evidence that oxidative stress is involved in the pathogenesis of glaucoma[13,18–20]. Oxidative stress results in deleterious molecular and cellular changes in the trabecular meshwork and hence aqueous humour outflow[18] and impacts RGCs, lamina cribrosa cells and optic nerve head astrocytes resulting in glaucomatous optic neuropathy[2,19–22]. Lipids, like polyunsaturated fatty acids (PUFAs), are specifically susceptible to ROS induced damage termed lipid peroxidation[23,24].

Unlike the stoichiometric ROS damage to proteins, carbohydrates and nucleic acids, lipid peroxidation can be initiated by a single reactive free radical leading to a chain reaction resulting in the oxidation of multiple PUFA residues and the generation of toxic metabolites[23,24], resulting in pathological cellular responses[25,26]. PUFAs contain two or more carbon-carbon double bonds, and are further classified by their carbon chain length and the position of the first double bond on the methyl terminal into two families: omega-3 (ω-3) and omega-6 (ω-6)[27]. Linoleic acid (LIN, ω-6) and alpha-linolenic acid (LNN, ω-3) are not synthesized in animals and are regarded as essential fatty acids[28]. Common PUFAs are EPA (eicosapentaenoic acid, ω-3) and DHA (docosahexaenoic acid, ω-3)[27,28]. PUFAs contain a high proportion of unsaturated bonds in their chemical structure which makes them liable to oxidative damage and lipid peroxidation[23,29]. ROS-mediated removal of bisallylic hydrogens generates free radicals which react with oxygen forming lipid radicals[29]. A solution to control the susceptibility of PUFAs to noxious lipid peroxidation reactions is by regioselective deuteration[29]. Deuterium is a stable hydrogen isotope that has natural abundance (150 ppm in ocean water) and is recognised by living systems as a normal, natural sub-type of hydrogen[30]. Deuterium incorporated into PUFAs at bis-allylic positions (D-PUFAs) inhibit the rate-limiting step of lipid peroxidation: ROS-driven hydrogen abstraction (hydrogen free radical is abstracted from a PUFA) from a bis-allylic site[29]. D-PUFAs causes the kinetic isotope effect[29], so that reactions involving cleavage of a C-H bond in PUFAs are slowed down in the C-D bond. As the abstraction step by ROS is repeated throughout the chain of lipid peroxidation events, the protective

effect of D-PUFAs is multiplied, thus resulting in a larger total beneficial effect when compared to exposure with normal PUFAs (H-PUFAs)[29]. D-PUFAs that are specifically deuterated at the bis-allylic positions, are resistant to lipid peroxidation[31–33] and can alleviate several pathologies associated with neurodegeneration [29,34].

Targeting oxidative stress in glaucoma is a potential therapeutic option to improve trabecular meshwork[35] and retinal ganglion cell function[36,37]. Decreasing the formation of lipid peroxidation products or scavenging them chemically could be beneficial in limiting the deleterious effects of oxidative stress in glaucoma. Post-mortem studies using retinal ganglion cells are challenging and so several patient derived cell samples have been used to explore the molecular basis of glaucoma and therapeutic responses including peripheral blood lymphocytes[38], dermal fibroblasts[39,40], trabecular meshwork[41] and ocular fibroblasts (Tenon's ocular fibroblasts)[42,43]. Tenon's ocular fibroblasts have been isolated from glaucoma patients with POAG and XFG to identify autophagy and mitochondrial dysfunction[43]. In this study, we utilised Tenon's ocular fibroblasts from patients with glaucoma (POAG) and non-glaucomatous controls to determine if isotopically reinforced D-PUFAs provide protection against oxidative stress and lipid peroxidation.

2. Materials and Methods

2.1. Patients

Participants with primary open angle glaucoma (POAG) and non-glaucomatous controls (patients undergoing cataract surgery) were recruited at the Royal Liverpool University Hospital, Liverpool, UK (Table 1). The study adhered to the tenets of Declaration of Helsinki and all participants gave informed written consent. Ethical approval for the study was acquired from the NHS Research Ethics Committee (REC Ref 14/LO/1088). Clinical phenotyping included a detailed ocular and medical history, drug history, intra-ocular pressure (IOP) measurement by Goldmann tonometry, slit-lamp biomicroscopy with stereoscopic disc examination and gonioscopy, and visual field testing (Humprey Visual Field Analyzer, Zeiss; Swedish interactive algorithm standard 24-2 program). The diagnosis of

POAG was based on open anterior chamber angles on gonioscopy, glaucomatous optic nerve damage on fundoscopy and a glaucomatous visual field defect. Patients were excluded if below 18 years of age, if they had previous intraocular surgery or any findings on examination suggesting ocular hypertension or a secondary cause of glaucoma. (e.g. pigment dispersion, pseudoexfoliative material in the anterior chamber, uveitis or ocular trauma). Ethnically matched and age matched controls without glaucomatous optic neuropathy and an IOP less than 21mmHg, were also recruited to the study as non-glaucomatous controls.

Table 1. Demographics and Clinical Data of Patients

Patient	Demographics	Diagnosis*	Presenting IOP#	C:D ratio‡	Glaucoma Severity
GTF#1	79 yrs old Female; Caucasian	POAG	RE: 28 mmHg LE: 28 mmHg	RE: 0.7 LE: 0.9	RE: Moderate LE: Advanced
GTF#2	81 yrs old Female; Caucasian	POAG	RE: 39 mmHg LE: 38 mmHg	RE: 0.8 LE: 0.7	RE: Advanced LE: Advanced
GTF#3	74 yrs old Female; Caucasian	POAG	RE: 22 mmHg LE: 24 mmHg	RE: 0.6 LE: 0.8	RE: Mild LE: Moderate
NTF#1	84 yrs old Female; Caucasian	Control	RE: 18 mmHg LE: 18 mmHg	Normal Normal	RE: Normal LE: Normal
NTF#2	86 yrs old Female; Caucasian	Control	RE: 12 mmHg LE: 13 mmHg	Normal Normal	RE: Normal LE: Normal
NTF#3	78 yrs old Female; Caucasian	Control	RE: 18 mmHg LE: 16 mmHg	Normal Normal	RE: Normal LE: Normal

GTF = Glaucomatous Tenon's ocular fibroblasts; NTF = Non-glaucomatous Tenon's ocular fibroblasts; * POAG = primary open-angle glaucoma (all cases were high tension glaucoma); # IOP = intra-ocular pressure and RE = right eye/LE = left eye; ‡ C:D ratio = cup:disc ratio.

2.2. Poly-unsaturated fatty acids (PUFAs)

Nondeuterated PUFAs (H-PUFAs: linoleic acid (LIN), linolenic acid (LNN), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterated poly-unsaturated fatty acids (D-PUFAs: D2-linoleic acid (D2-LIN), D4-linolenic acid (D4-LNN), D8-eicosapentaenoic acid (D8-EPA) and D10-docosahexaenoic acid (D10-DHA)) were prepared as described previously[31] and used as free acids (Figure 1).

2.3. Tenon's ocular fibroblasts cell culture

Human primary Tenon's ocular fibroblasts (TFs) were cultured from subjects with POAG (GTF) or non-glaucomatous controls (NTF) undergoing glaucoma or cataract surgery using the explant method as previously described[44]. The Tenon's tissue was harvested using the wound created for sub-Tenon's anaesthesia. This Cells were maintained in (Dulbecco's Modified Eagle's Medium/ Nutrient Ham F12 (1:1) medium (DMEM/F12) (Sigma Aldrich, UK) supplemented with L-glutamine (Sigma-Aldrich, UK), 10% fetal calf serum (Sigma-Aldrich, UK), penicillin/streptomycin mix (1:1) (Sigma-Aldrich, UK) and amphotericin (Sigma-Aldrich, UK). Samples were incubated at 37°C (5% CO_2 and 95% humidity). The fibroblasts were sub-cultured once they reached 70% confluency to generate multiple sub-cultures which were used up to passage 4. TF characterisation was carried out as previously described[45]. Briefly, cell morphology was assessed using light microscopy and cultured TFs were fluorescently stained with the fibroblast marker vimentin (V9) mouse monoclonal antibody (Thermofisher Scientific, USA) and counter stained with DAPI (Abcam, USA) for nuclear visualisation (Figure 2A and B).

2.4. Optomisation of menadione-induced ROS

To study the therapeutic effects of PUFAs against ROS in NTFs and GTFs, we induced intracellular ROS using menadione (Sigma-Aldrich, UK). Menadione is a quinone that is metabolized by an electron reducing enzyme generating intracellular superoxide therefore increasing intracellular ROS[46]. TFs were plated at 1x105 in a 96 well plate and allowed to attach. NTF and GTF cells were serum starved overnight before stimulation with menadione using concentrations ranging from 0-100µM for 24hrs. Cytotoxicity was assessed using the MTT cell viability assay (Sigma-Aldrich, UK) and ROS levels were assessed using the CellROX green probe (ThermoFisher Scientific, UK).

2.5. Cell viability assay

Cell viability was assessed using the MTT colorimetric assay (Sigma-Aldrich, UK). TFs were plated at 1x105 in a 96 well plate and allowed to attach. Cells were serum starved overnight. Oxidative stress was induced by treating the cells with 15µM of menadione for 24hrs. Cells were treated with various doses of H- and D-PUFAs for 24hrs post menadione induction. MTT was added to the wells and incubated for one hour. Formazan crystals were solubilised in DMSO and quantified using a plate reader at 570nm (Omega Starlabs; UK).

2.5. ROS assay

The level of intracellular ROS was measured using the CellROX green probe (ThermoFisher Scientific, UK). This is a cell-permeant dye exhibits bright green fluorescence when oxidised by ROS. TFs were plated at 1x105 in a 96 well plate and allowed to attach. Oxidative stress was induced using 15µM of menadione for 24hrs. PUFAs were added post menadione induction for a further 24hrs. CellROX green was added directly to the media (final concentration 5µm) and incubated for 30minutes. Cells were washed 3x with PBS and the ROS accumulation was determined by measuring the levels of CellROX green using a plate reader (Omega Starlabs; Ex/Em 485/528 nm). The mean signal intensity (in relative fluorescence units [RFU]) for each sample was calculated and averaged.

2.6. Lipid peroxidation assay

Malondialdehyde (MDA) levels were measured by using the Lipid Peroxidation Assay Kit (ab118970; Abcam, Cambridge, UK). TFs were homogenized on ice in 303 µL MDA lysis buffer and centrifuged at 14,000×g for 10 min at 4 °C. This was followed by 200 µL of the supernatant being mixed with 600 µL of the TBA solution, incubated at 95 °C for 60 min and then cooled on ice for a further 10 min, after which 200 µL of the mixed solution was added to a 96-well microplate. The MDA level was calculated with standard curve at a wavelength 532 nm and calculated following the manufacturer's protocols.

2.7. Statistical analysis

All the data was expressed as the mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis being performed using GraphPad Prism 5.0. p value < 0.05 was considered as statistically significance. Three independent biological donors (glaucoma and non-glaucomatous) were used in each experiment.

3. Results

3.1. The induction of oxidative stress in Tenon's ocular fibroblasts.

To compare the levels of oxidative stress in the NTFs and GTFs, we cultured the TF cells for 24hrs and probed with CellROX green. The basal oxidative stress levels were significantly elevated in GTFs compared to NTFs (Figure 2C). To simulate the oxidative environment of glaucoma, we subjected NTF and GTF cells to menadione at concentrations ranging from 0-100μM for 24hrs. Menadione induced toxicity was assessed using an MTT assay and demonstrated a dose dependent reduction in cell viability in both NTFs and GTFs (Figure 2D). To establish an appropriate menadione dose to induce oxidative stress in both NTFs and GTFs, cells were subjected to menadione (0-100μM) for 24hrs and probed using CellROX green (Figure 2E). Menadione induced a dose dependent increase in ROS levels with the GTF showing a consistent exaggerated response compared to NTF. A dose of 15μM of menadione was chosen for subsequent experiments as it was the least toxic while still significantly inducing oxidative stress beyond basal ROS levels.

3.2. The effects of the normal and modified PUFAs on menadione reduced cell viability in Tenon's ocular fibroblasts

The MTT colorimetric assay was used to establish if unmodified PUFAs and D-PUFAs at doses of 25, 50 and 100μM can rescue the menadione (15μM) induced reduction in NTF and GTF cell viability over 24 hours. LIN failed to rescue menadione-reduced cell viability, however, at 25μM and 50μM

D2-LIN rescued and increased the viability in both the NTFs and GTFs; however this was only significant in GTFs at a dose of 50µM for D2-LIN (Figure 3A). Both LNN and D4-LNN improved cell viability at concentrations of 25µM and 50µM (Figure 3B). Similarly, EPA and D8-EPA improved cell viability, however, a significant improvement was only achieved at 50µM and 100µM with EPA while the modified D8-EPA form significantly rescued viability even at the lower 25µM concentration (Figure 3C).

3.3. The effects of the normal and modified PUFAs on menadione induced oxidative stress in Tenon's ocular fibroblasts

To investigate whether the PUFAs can modulate intracellular ROS formation, both NTFs and GTFs were incubated with menadione (15µM for 24 hours) and then treated with unmodified PUFAs and D-PUFAs. The deuterated D2-LIN, D8-EPA and D10-DHA has a more profound effect in reducing ROS levels compared to the unmodified PUFAs in NTFs (Figure 4A). Unmodified PUFAs had limited impact on rescuing menadione induced ROS in GTFs whereas D8-EPA and D10-DHA reduced ROS with D8-EPA showing the most marked rescue (Figure 4B). Both unmodified and D-PUFAs forms of LIN and LNN failed to induce a therapeutic effect in GTFs. H-DHA failed to decrease oxidative stress levels in both NTF and GTF donors (Figure 4).

3.4. PUFAs reduce menadione-induce lipid peroxidation in Tenon's ocular fibroblasts

We used MDA levels as a marker of oxidative lipid damage as MDA is the final toxic products of polyunsaturated fatty acids peroxidation in the cells. Menadione (15µM for 24 hours) significantly increased the level of MDA in both the NTFs (0.185 vs 0.763 nmol/ml; Figure 5A) and GTFs (0.262 vs 0.732nmol/ml; Figure 5B) compared to the vehicle control. Both LIN and D2-LIN showed equivalent effects reducing MDA levels in NTFs (21% and 30% reduction) and GTFs (16% and 23% reduction). In

both NTFs and GTFs, the D2-LIN was more potent than the non-modified LIN PUFA. In the NTFs, only the D4-LNN form significantly reduced MDA levels by 17%, while in the GTFs, both LNN and D4-LNN reduced MDA levels. Both EPA and D8-EPA reduced MDA levels in NTFs by 21% and 41% respectively; and GTFs a reduction of 27% and 40% was observed. In both NTFs and GTFs, the D8-EPA was more potent than the non-modified EPA form. Both the DHA and D10-DHA reduced MDA levels by 16% in the NTF's, whereas only the D10-DHA significantly reduced the MDA levels in the GTFs. Overall, D8-EPA produced the most profound reduction in MDA and lipid peroxidation in both NTFs and GTFs.

4. Discussion

We have shown that Tenon's ocular fibroblasts from glaucoma patients have significantly increased basal oxidative stress than non-glaucomatous Tenon's ocular fibroblasts. Furthermore, we have shown that deuterated polyunsaturated fatty acids (D-PUFAs) provide an enhanced rescue of menadione induced lipid peroxidation in both NTFs and GTFs using malondialdehyde (MDA) levels as a marker. Malondialdehyde (MDA) is one of the final toxic products of polyunsaturated fatty acids peroxidation in the cells and widely used as a marker of oxidative lipid damage[23,25]. MDA was a consistent biomarker of lipid peroxidation and oxidative stress in a meta-analysis of 22 case-control glaucoma studies[13], is evaluated in the aqueous humour of glaucoma patients[47–49] and increased in the trabecular meshwork from POAG patients[50]. In patients with pseudo-exfoliation glaucoma (XFG) oral supplementation with DHA/EPA with antioxidants for 6 months significantly reduced serum MDA levels and oxidative stress[51].

Multiple clinical and experimental studies have implicated oxidative stress in the pathogenesis of glaucoma[2,13–18,21,22,47,52]. Fatty acid oxidation is the major oxidation pathway in glaucoma[53]. Current glaucoma treatments target intra-ocular pressure but decreasing the formation of lipid peroxidation products or scavenging them chemically could represent a novel

therapeutic strategy to limit the deleterious effects of oxidative stress in glaucoma. The dietary intake and supplementation of PUFAs has been associated with numerous positive health benefits including prevention of cardiovascular disease and control of inflammatory diseases[27,28]. Clinical studies have shown that unmodified long chain PUFAS have pleiotropic actions including neuroprotection[54], anti-inflammatory[55] and antioxidant[56,57] effects which can modulate the molecular pathways involved in the pathogenesis of ocular conditions, such as, age-related macular degeneration[58], diabetic retinopathy[59] and retinal diseases[60,61]. In fact, omega 3 PUFAs reversed H2O2 -induced glaucomatous effects in human primary trabecular meshwork cells and provided support for the concept of PUFA prevention of oxidative stress in glaucoma[62].

Our study shows that EPA exhibited a therapeutic effect against menadione induced oxidative stress in NTFs and GTFs by rescuing cell viability, reducing oxidative stress and reducing MDA levels (lipid peroxidation). We found that all four D-PUFAs reduced the levels of MDA in both the NTFs and GTFs, but that the reduction of MDA was amplified by EPA deuteration. Our data also shows the addition of both the unmodified and D-forms of LIN, LNN and EPA improved cell viability in both the NTFs and GTFs. Consistently, unmodified and D-modified LIN and EPA exhibited a superior biological effect against menadione induced oxidative damage in TFs. LIN exhibits poor chemical stability rendering it liable to autoxidation[63]; therefore, making it the ideal candidate for deuteration. In yeast cells D2-LIN reduced autoxidation and oxidative stress induced lipid peroxidation[31]. Currently the use of an isotope-reinforced D-LIN is in clinical use in neurodegenerative diseases[34,64,65]. Our study using Tenon's ocular fibroblasts from glaucoma patients suggests that deuterated PUFAs, specifically D2-LIN and D8-EPA, may provide a potentially safe and effective method to reduce the cytotoxic oxidative stress in glaucoma.

Patients with POAG show abnormalities in blood fatty acid composition with reduced levels of EPA[66] and DHA[66,67] ; and interestingly the level of DHA reduction was greater in POAG patients with more severe disease[66,67]. DHA depletion was also associated with red blood cell membrane

rigidity which was proposed as a potential factor impacting optic nerve perfusion exacerbating glaucomatous optic neuropathy[66,67]. Observational studies have demonstrated that omega 3 fatty acids intake have a protective effect against POAG[68] and normal tension glaucoma[69]. Human epidemiological studies have investigated dietary PUFA intake and glaucoma. In the US National Health and Nutrition Examination Survey (NHANES) an increased DHA and EPA and global omega 3 intake decreased the risk of glaucoma[70]. However, higher levels of consumption of total PUFAs were associated with a higher risk of glaucoma and the authors proposed that increasing the proportion of omega 3 PUFAs while controlling total daily PUFA intake may be protective against glaucoma[70]. Similarly, the relative proportion of omega 3 to omega 6 has also been associated with glaucoma particularly high tension POAG[71].

In animal studies omega-3 (ω-3) reduce intra-ocular pressure (IOP) via the production of a docosanoid precursor (DHA) increasing aqueous humour outflow[72]. Oral omega-3 (ω-3) PUFAs (LNN, EPA and DHA) treatment for 3 months significantly reduced IOP in normal human subjects[73]. In patients with pseudo-exfoliation glaucoma (XFG) oral supplementation with DHA/EPA (omega 3) with antioxidants for 6 months reduced IOP[51]. Beyond lowering IOP omega 3 fatty acid intake showed neuroprotective effects in animal models of glaucoma[74–77]. Omega 3 fatty acids are essential for retinal ganglion cell (RGC) function[74,75] and RGCs show high ω-3 uptake in tracer studies[78,79]. In rats dietary deficiency of omega-3 (ω-3) alone[75] and dietary deficiency of omega-3 (ω-3) plus IOP insult are additive in the induction of retinal ganglion cell dysfunction[74]. In a laser-induced rat model of raised IOP a 6-month diet supplemented with a combination of EPA, DHA, and gamma-linolenic acid preserved retinal structure damage and decreased glial cell activation[77]. Supplementation of omega 3 (ω-3) PUFAs had a neuroprotective effect in the glaucomatous degeneration seen in the DBA/2J mice and this effect was enhanced with concomitant topical timolol beta blocker eyedrops[76].

In human studies of the retinal and RGC effects of PUFAs, combined oral DHA, vitamin E and vitamin B complex supplementation improved visual field indices and contrast sensitivity in a small series of POAG patients[80] and omega 3 (DHA/EPA) supplementation also improved blue/yellow perimetric indices in patients with ocular hypertension[81]. However, an open-label randomized controlled trial on 117 patients with mild or moderate POAG and IOP under control with topical medications failed in reporting any benefit after oral antioxidant supplementation with or without omega-3 PUFAs[82]. This might not be unexpected given the trial was performed in a relatively small number of cases with controlled mild to moderate POAG with a study duration of only 6 months given the well-recognised challenges in performing neuroprotection studies in glaucoma. Using D-PUFAs would also have additional therapeutic benefits in glaucomatous neurodegeneration[29].

5. Conclusions

In PUFAs, replacement of the bis-allyic hydrogen atoms with deuterium, a heaver and more stable isoform, results in stabilisation of the C-H bond[29,31,81]. This bond is initially broken in the lipid peroxidation process, initiating the formation of lipid peroxidases and activation of the toxic cellular cascades. Once this cascade is activated, the radicals produced can attack other PUFAs in the lipid membrane and increase electrophilic stress[29,31,83]. Electrophilic stress has been implicated in aging, cardiovascular and neurodegenerative diseases[84]. Previous studies have shown that substitution with as little 20% with deuterated PUFAs in comparison to normal PUFAs can exert a protective effect and impede the process of the chain reaction[83,85]. In the present study we have shown that although PUFAs are protective against menadione induced oxidative stress in TFs, modified isotope-reinforced D-PUFAs exert a superior effect by improving cell viability and reducing oxidative stress and lipid peroxidation.

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Conflicts of Interest

The authors declare no conflict of interest. D-PUFAs were supplied by Retrotope, Inc., Los Altos, CA, USA) for supplying the D-PUFAs used in the study but had had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. M.S. Shchepinov is employed by and holds stock in Retrotope, Inc.

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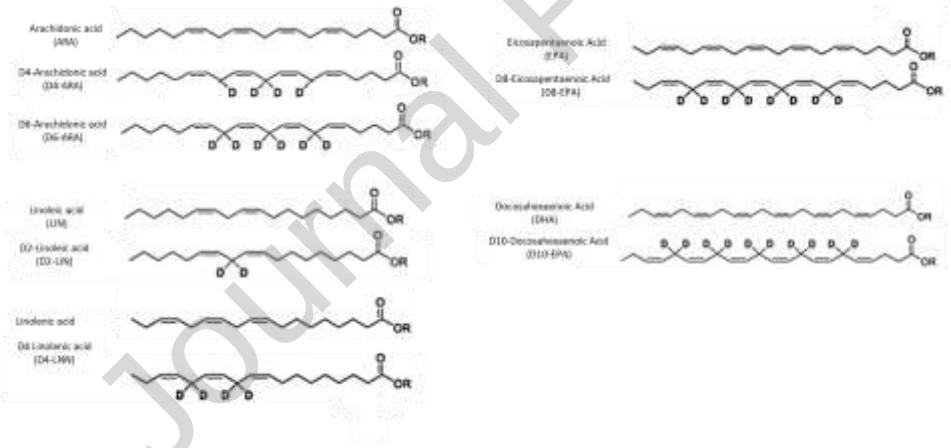
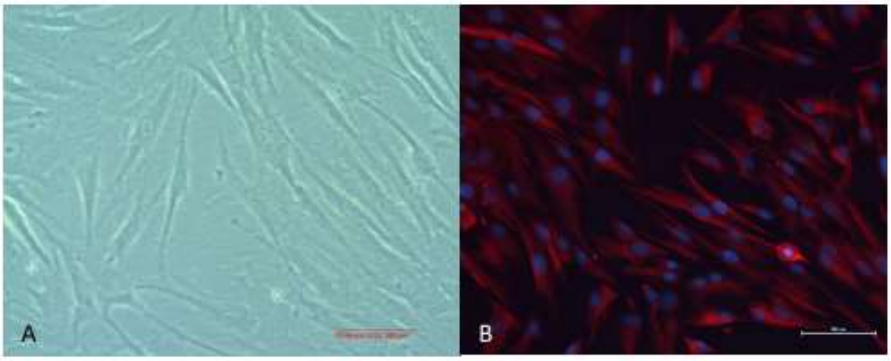
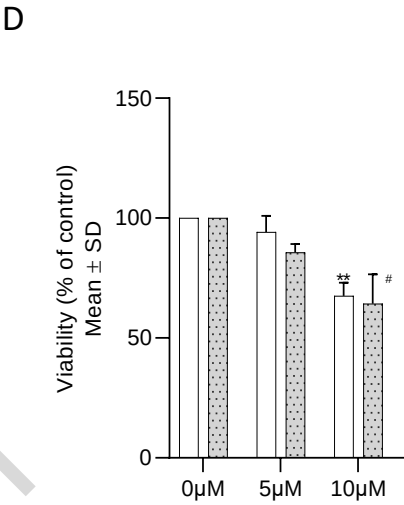
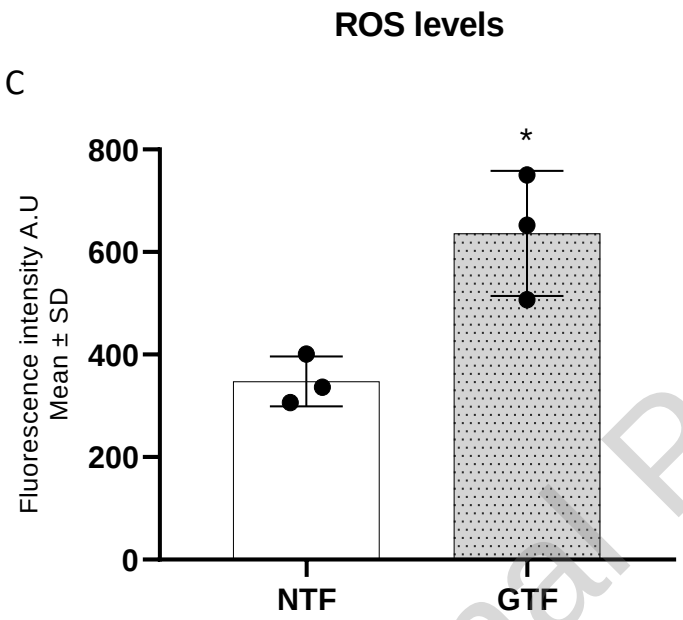


Figure 1: Structures of polyunsaturated fatty acids (PUFAs) used in this study. Linoleic acid (LIN) and D2 linoleic acid (D2-LIN); linolenic acid (LNN) and D4 linolenic acid (D4-LNN); eicosapentaenoic acid (EPA) and D8 eicosapentaenoic acid (D8-EPA); and docosahexaenoic acid (DHA) and D10-docosahexaenoic acid (D10-DHA).







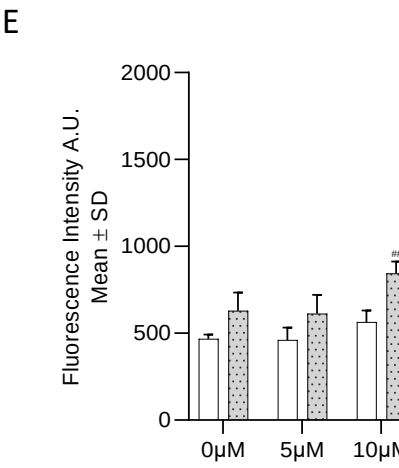


Figure 2. Oxidative stress induction in Tenon's ocular fibroblasts. Characterisation of Tenon's ocular fibroblasts using bright focus microscopy (A) and fluorescent vimentin staining (B). Glaucomatous Tenon's ocular fibroblasts (GTF) have higher basal levels of reactive oxygen species (ROS) compared to non-glaucomatous Tenon's ocular fibroblasts (NTF) (C). Menadione dose dependently reduces cell viability in both NTF and GTF (D) and increases ROS levels. Values represent the mean of three independent biological samples for NTFs and GTFs. Statistical significance was determined using Student T- tests and a one-way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus untreated non-glaucomatous TF cells.

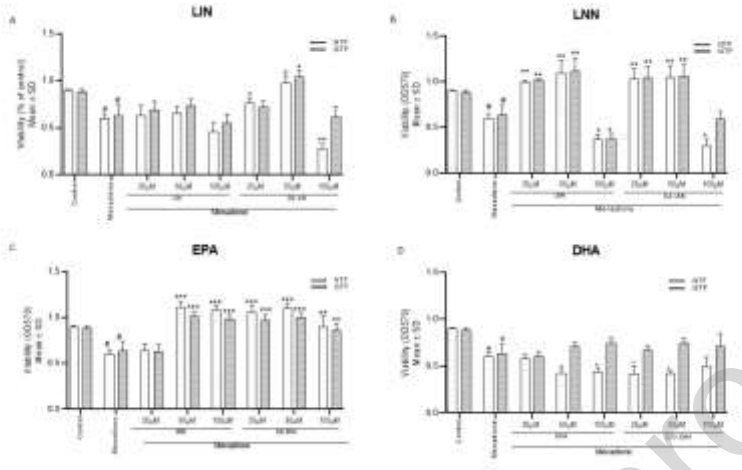


Figure 3. PUFAs improves the viability of menadione induced toxicity in Tenons ocular fibroblasts. Cell viability (MTT assay) of non-glaucomatous and glaucoma Tenon's ocular fibroblasts (NTF and GTF respectively) incubated with menadione (15µM) for 24hrs followed by treatment with different concentrations of normal and modified (A) linoleic acid (LIN and D2-LIN), (B) linolenic acid (LNN and D4-LNN), (C) eicosapentaenoic acid (EPA and D8-EPA), and (D) docosahexaenoic acid (DHA and D10-DHA). Values represent the mean of three independent biological samples for NTFs and GTFs. Statistical significance was determined using a one-way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus menadione treated cells. # $p < 0.05$, ## $p < 0.01$, versus non-glaucomatous TF cells.

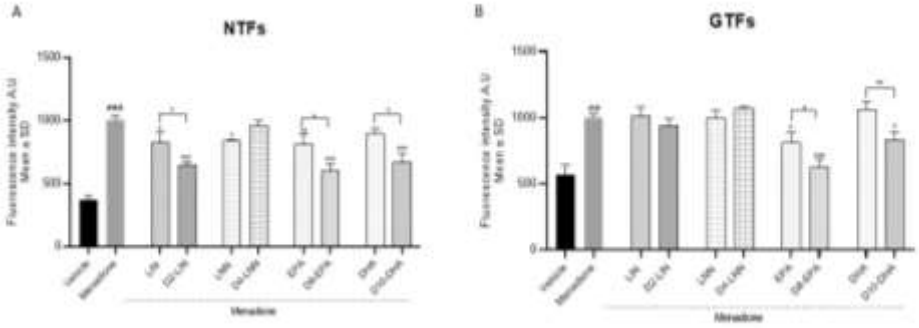


Figure 4. PUFAs reduce levels of reactive oxygen species in menadione induced Tenon's ocular fibroblasts. ROS levels were used as a marker of oxidative stress and quantified using CellROX green. Non-glaucomatous ocular fibroblasts (NTFs; A) and glaucoma Tenon's ocular fibroblasts (GTFs; B) fibroblasts were incubated with menadione (15µM) for 24hrs followed by treatment with 50µM of normal and deuterated linoleic acid (LIN; D2-LIN), linolenic acid (LNN; D4-LNN), eicosapentaenoic acid (EPA; D8-EPA) and docosahexaenoic acid (DHA; D10-DHA). Values represent the mean of three independent biological samples for NTFs and GTFs. Statistical significance was determined using a one-way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus menadione treated cells. # $p < 0.05$, ## $p < 0.01$, non-glaucomatous TF cells.

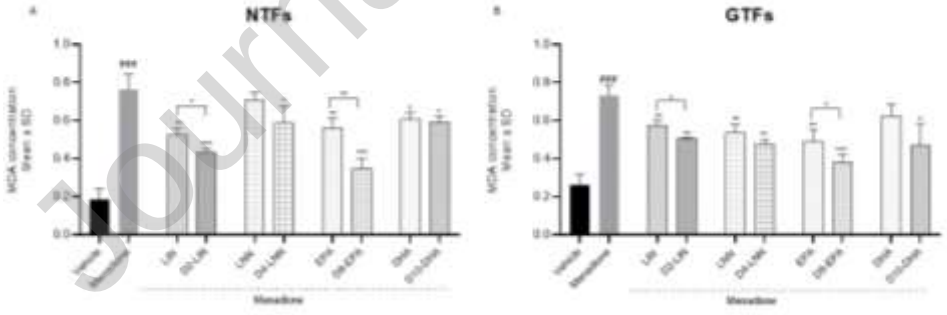
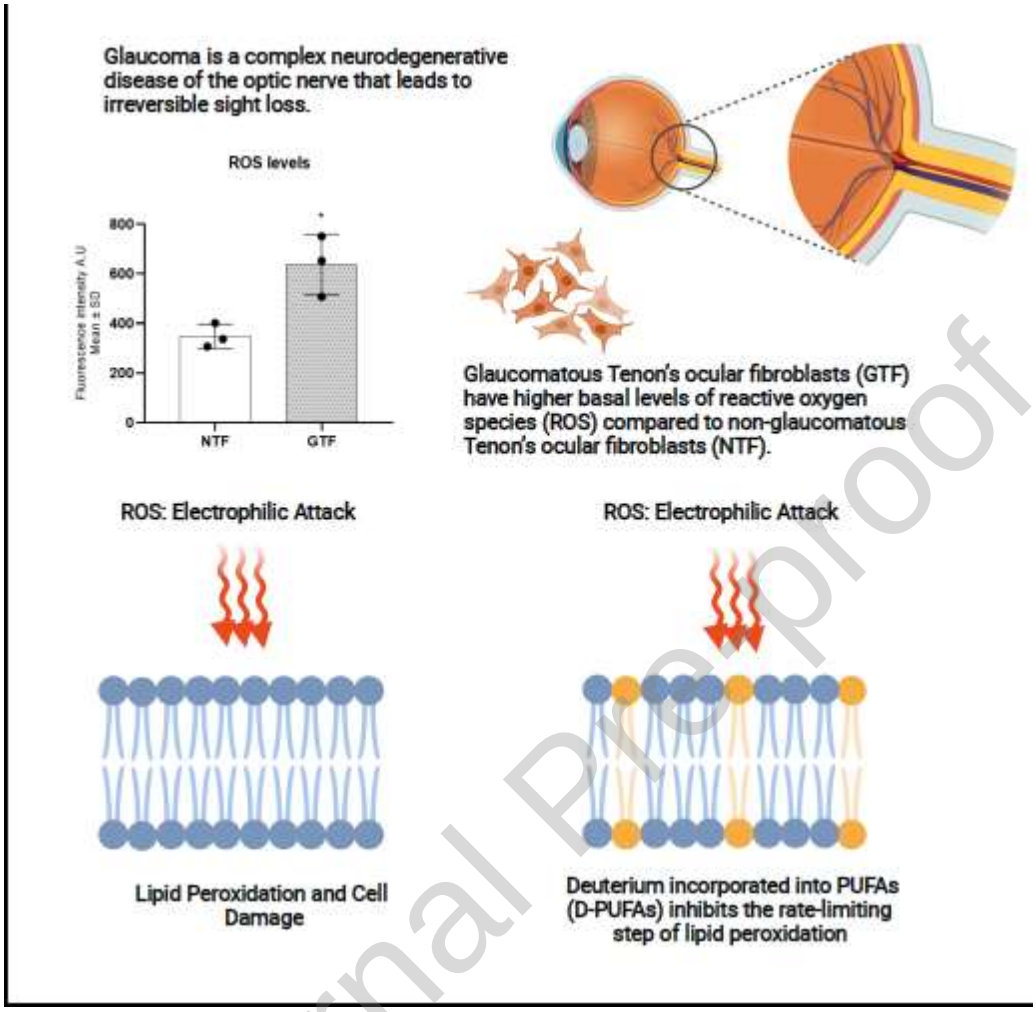


Figure 5: Deuterium modified PUFAs reduce menadione induced lipid peroxidation in non-glaucomatous and glaucomatous Tenon's ocular fibroblasts. Non-glaucomatous Tenon's ocular fibroblasts (NTF) (A) and glaucomatous Tenon's ocular fibroblasts (GTF) (B) were incubated with menadione (15µM) for 24hrs followed by treatment with 50µM of normal and modified linoleic acid (LIN and D2-LIN), linolenic acid (LNN and D4-LNN), eicosapentaenoic acid (EPA and D8-EPA) and

docosahexaenoic acid (DHA and D10-DHA). Lipid peroxidation levels were assessed by quantification of malondialdehyde (MDA) content. Values represent the mean of three independent biological samples for NTFs and GTFs. Statistical significance was determined using a one-way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus menadione treated cells. # $p < 0.05$, ## $p < 0.01$, non-glaucomatous TF cells.

Graphical abstract



Highlights

- Glaucoma is a complex neurodegenerative disease that leads to irreversible sight loss.
- Oxidative stress is involved in the pathogenesis of glaucoma.
- Oxidative stress was elevated in glaucomatous Tenon's ocular fibroblasts.
- Isotopically reinforced D-PUFAs provide protection against lipid peroxidation.