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# Identification and quantification of ionising radiation-induced oxysterol formation in membranes of lens fibre cells

Running title: Ionising radiation induced oxysterol formation in the eye lens

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Passed away June 4th 2019. This article is dedicated to his memory.

Running title: Ionising radiation induced oxysterol formation in the eye lens

The HIGHLIGHT for the article is suggested to be as follows:

7β-hydroxycholesterol and 7-ketocholesterol both correlate with lens cataract

Ionising Radiation (IR) exposure increases oxysterols in isolated lens membranes

Mouse exposure to low dose IR, increases oxysterols in the living lens
Oxysterol increase is dose dependent and transient in living lenses
Oxysterol levels higher in older compared to younger lens cells

Abbreviations: DBSs, DNA double strand breaks; IR, ionising radiation; LECs, lens epithelial cells; LFC, lens fibre cells; ROS, reactive oxygen species; BoC, Bovine cortex; BoN, Bovine nucleus; MoC, Mouse cortex; MoN, Mouse nucleus; PTMs, post-translational modifications; RT, room temperature; n.s., not significant; h, hours

## ABSTRACT

Ionising radiation (IR) is a cause of lipid peroxidation, and epidemiological data have revealed a correlation between exposure to IR and the development of eye lens cataracts. Cataracts remain the leading cause of blindness around the world. The plasma membranes of lens fibre cells are one of the most cholesterol rich membranes in the human body, forming lipid rafts and contributing to the biophysical properties of lens fibre plasma membrane. Liquid chromatography followed by mass spectrometry was used to analyse bovine eye lens lipid membrane fractions after exposure to 5 and 50 Gy and eye lenses taken from whole body 2 Gy irradiated mice. Although cholesterol levels do not change significantly, IR dose-dependent formation of the oxysterols 7ϐ-hydroxycholesterol, 7-ketocholesterol and 5, 6-epoxycholesterol in bovine lens nucleus membrane extracts was observed. Whole body X-ray exposure (2 Gy) of 12-week old mice resulted in an increase in 7ϐ-hydroxycholesterol and 7-ketocholesterol in their eye lenses. Their increase regressed over 24 hours in the living lens cortex after IR exposure. This study also demonstrated that the IR induced fold increase in oxysterols was greater in the mouse lens cortex than the nucleus. Further work is required to elucidate the mechanistic link(s) between oxysterols and IR-induced cataract, but these data evidence for the first time that IR exposure of mice results in oxysterol formation in their eye lenses.

Keywords: cholesterol, X-rays, ionising radiation, eye lens, oxysterols, age-related cataract

## INTRODUCTION

The eye lens focuses light onto the retina and therefore its transparency and optical function is essential to vision [1]. Opacity and visual impairment are the main clinical characteristics presented in eye lens cataract. As the lens is a system where proteins [2, 3] and lipids [4, 5] are retained throughout life, post-translational modifications and oxidative and damage accumulate in these biomolecules with increasing age (reviewed in [1]). Cataracts are an iconic age-related pathology, but epidemiological data suggest that they can also be caused by exposure to ionising radiation (IR) because there is a clear correlation between ionising radiation (IR) exposure and cataractogenesis [6-8]. The mechanisms involved are still under investigation [9], but IR damages macromolecules either directly or indirectly by ionising water into free radicals to then cause lipid, protein and DNA damage. Such damage will collectively contribute to the cataractogenic load upon the lens, which is defined as the biomolecular damage accumulated over a lifetime as a result of lifestyle, genetic and environmental events [10]. Significant advances have been made regarding the effects of IR on DNA and proteins in the eye lens (reviewed by [10]). The IR effect(s) upon cholesterol, the most abundant lipids in the lens cell membranes [11], has not been investigated. Free radicals cause cholesterol oxidation [12-14] and IR exposure leads to oxysterol formation [15] in a dose dependent manner [16], which are involved in many other age-related diseases [17] including cataract [18, 19].

Doses >0.5 Gy have been shown to cause irreparable DSBs and change the organisation, differentiation and proliferation of lens epithelial cells (LECs) [20-22]. Low dose IR exposure, including doses <0.5 Gy, changes cell proliferation and consequently cell density as well [23]. IR exposure stimulates protein post-translational modifications and aggregation resulting in loss of transparency and cataract formation in the eye lens [24-27]. IR would be expected to cause oxysterol formation in the lens, but this remains to be demonstrated. This is important because there is a strong correlation between the presence of oxysterols and cataract formation [18, 19] and this is true also for other age related eye diseases such as macular degeneration [17]. Interestingly, Smith-Lemli-Optiz syndrome also has cataract as a phenotype [28] and this is due to a deficiency in 7-dehydrocholesterol reductase that causes a build-up of 7-dehydrocholesterol, a lipid that is particularly sensitive to free radical oxidation [14, 28]. This is further evidence that oxysterols are involved in cataract formation and other inborn errors of metabolism affecting cholesterol metabolism have also been linked to cataract [29, 30].

The eye lens comprises a single layer of LECs covering the anterior hemisphere of the lens, and lens fibre cells (LFCs) that differentiate from LECs form the major part of the lens. During differentiation, all cell organelles in the LFCs are degraded and the cytoplasm is filled with crystallins [31]. The youngest LFCs are at the lens cortex, and the oldest cells are situated at the centre of the lens, which is known as the nucleus. The LFCs plasma membrane is one of the most cholesterol rich membranes in the body; it also contains dihydrosphingomyelin as the most abundant phospholipid in humans [11, 32]. With age, the levels of dihydrosphingomyelin and cholesterol rise, even crossing the cholesterol saturation limit, leading to membrane lipid raft formation. In contrast, the levels of glycerolipids are found be declined due to the preferential oxidation of glycerophospholipids [33-36]. Electron paramagnetic resonance analyses showed the molar ratio of cholesterol:phospholipids was up to 4:1 in the human nucleus [37].

In addition to mediating the organisation and function of integral membrane proteins [38-41], a protective role for the high cholesterol levels against cataract has been suggested due to the decreased oxygen permeability and subsequent reduced oxidative stress in bovine and human eye lenses [34, 42-45]. Cholesterol:phospholipid molar ratio measurements demonstrated decreased cholesterol levels in cataractous lenses [37, 46]. Moreover, patients with defects in genes coding for essential enzymes in the cholesterol synthesis pathway, such as 7-dehydrocholesterol reductase in SmithïLemliïOpitz syndrome [47], mevalonate kinase in mevalonic aciduria [48] and lanosterol synthase [49], show a high incidence of cataract, emphasising the importance of cholesterol homeostasis in lens transparency. Mutations in CYP21A2, a 21 hydroxylase needed to metabolise cholesterol to cortisol and aldosterone has recently been found to cause autosomal dominant cataract [30] as is also the case for CYP27A1 [50]. In animal models, drugs that interfere with cholesterol biosynthesis also cause cataract [51].

Ageing is a major risk factor for cataract formation because of the time-dependent increase in oxidative stress, metabolic ageing (deleteriome; [52]) and loss of reducing potential in the lens [10]. Over time the anti-oxidant defences in the lens become less efficient because of the development of a glutathione barrier between the lens cortex and nucleus [53, 54]. Consistent with this concept, a cholesterol oxidation adduct, 7-keto cholesterol, builds up with age in the lens [55]. Cholesterol oxidation products, i.e. 20Ŭ-hydroxycholesterol, 25-hydroxycholesterol, 7ƀ-hydroxycholesterol, 7-ketocholesterol and 5, 6-epoxycholesterol, have been found to accumulate in human lenses with cataract as compared with age-matched

clear lenses [18]. These oxysterols can be formed via enzymatic or non-enzymatic (autoxidation) processes depending on enzyme availability and the type of reactive oxygen species (ROS) causing the oxidative stress [56-58]. Given that IR elevates oxidative stress in the eye lens, and oxysterol content correlates with age-related cataract (ARC), investigation of the effects of IR exposure on the lipid membrane of LFCs was performed. ARC-associated oxysterols were identified when isolated bovine lens membranes were exposed to X-rays (5 ï 50 Gy). The formation of these oxysterols *in vivo* following whole body exposure of mice to 2 Gy X-rays was also demonstrated and their levels monitored over a 24 h time period. These data revealed that after an initial increase, oxysterol levels decayed in membrane fractions isolated from the lenses IR-exposed mice.

## MATERIALS AND METHODS

### Bovine LFC membrane fraction preparation

Bovine eyes were obtained from Linden Burradon Food Supply (FSA approved Scientific Research Material collection No. 2056). To collect the cholesterol enriched lens membranes, a protocol used to purify lens membrane fractions [59] was adapted. Decapsulated eye lenses were aqua-dissected by stirring them in a low salt phosphate buffer (10 mM $Na_2HPO_4$ [pH 7.4], 100 mM NaCl and 5 mM EDTA [pH 8.0]) to first collect the cortical fraction of the LFCs followed by the nuclear fraction. A series of buffer extractions designed to enrich for integral membrane proteins and the lipid membranes was performed [60]. The LFC membranes were pelleted by centrifugation (31,000×g @ $r_{max}$ at 4°C for 20 min (Beckman JA20 rotor). Membranes were purified using the following buffer extraction sequence; a high salt buffer (10 mM $Na_2HPO_4$ [pH 7.4], 1.5 M KCl, 5 mM EDTA [pH 8.0]), an ammonium bicarbonate buffer (100 mM $NH_4HCO_3$, 1 mM EDTA [pH 8.0]), a urea buffer (10 mM $Na_2HPO_4$ [pH 7.4], 8 M Urea, 5 mM EDTA [pH 8.0]) and lastly a sodium hydroxide buffer (100 mM NaOH), with a low salt phosphate buffer washes in between each step. The final cortical (BoC) and nuclear (BoN) membrane fractions were resuspended in the low salt phosphate buffer and stored at 4 °C until required.

### Exposure of membrane fractions to X-rays

The BoC and BoN fractions (10 mg wet weight) were resuspended in 1 mL of the low salt phosphate buffer and exposed to 5 and 50 Gy in a single X-ray dose using an X-ray chamber irradiator calibrated to national standard (IRR320, aluminium filtered 320 kV, dose rate 5 Gy/min). EDTA is expected to scavenge free radicals, but a fraction of the water molecules is bound to the phosphocholine headgroups [61] as well as permeating the AQP0 water

channels in these lens membrane fractions[62]. Dose delivery was verified at the exact site where the samples were placed with aluminium oxide chips [63].

### Mouse irradiation and lens membrane fraction preparation

Female mice C57BL/6JOla/Hsd (C57BL/6J) were obtained from Envigo RMS (UK) Ltd. (Blackthorn, Bicester, Oxfordshire OX25 1TP) and were housed in groups of four. Food (RM3(E), LBS technology) and water were provided *ad libitum*. All procedures involving mice were performed according to the UK Animals (Scientific Procedures) Act 1986, and ethical approval was obtained from the United Kingdom Home Office and the local Animal Welfare and Ethical Review Body at the UK Health Security Agency. At 3 months of age, female mice were exposed to 100 mGy or 2 Gy in a single X-ray dose (CD160/1, AGO X-ray Ltd., aluminium and copper filtered (~1 mm) containing a Varian NDI-320 source; 250 kVp; dose rate 0.5 Gy/min). Dosimetry was performed with a calibrated reference ionisation chamber for the exact exposure setup used. The monitoring of the exposures was accomplished with a calibrated UNIDOS E electrometer and ó in-beamô monitor ionisation chamber (PTW). To verify whether the dose was delivered to the entire area of the box, spatial dose uniformity was measured with Gafchromic EBT2 films (Vertec Scientific Ltd.). Subsequently, the mice were returned to their cages and received standard care until sacrifice at 2h, 24 h and 7 days post-irradiation allowing for 4 mice per time point.

Eight lenses were removed and processed together essentially as described above for bovine LFC membrane preparations using the following buffer sequence: low phosphate, urea, low phosphate and sodium hydroxide buffer. Mouse LFC membranes were pelleted by centrifugation (17,000×g @ $r_{max}$ at 4°C for 20 min (Eppendorf refrigerated microfuge). The final cortical (MoC) and nuclear (MoN) membrane fractions were resuspended in the low salt phosphate buffer and kept at 4 °C until further analysis.

For the measurement of cholesterol and 7 keto-cholesterol in 6 and 30 month mouse lens samples, LFCs were also prepared from wild type C57BL/6J mice.

### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were resuspended in a SDS buffer (1 mM EDTA pH 7.8, 50 mM Tris pH 6.8 and 1% (w/v) SDS) and then mixed with 4x denaturing sample buffer (containing 2.854 M ß-mercaptoethanol) ([64] as modified in [59]). Equal protein loadings on the gel and samples were separated on 15% (w/v) polyacrylamide gels at 100 V. Proteins were visualised by Coomassie blue staining (0.25% (w/v) Coomassie Brilliant Blue R250, Merck).

### Detection of carbonylated proteins

Carbonylated proteins were detected using the OxyBlot Protein Oxidation Detection Kit (Merck) following the manufacturerôs instructions.

### Lipid purification

Lipids were purified using a modified Bligh-Dyer method [65] and oxysterols using published procedures [66]. All glassware was rinsed with methanol and hexane and all extraction solvents were sparged with nitrogen for 10 min. The bovine lens membranes (10 mg of wet weight) or mouse lens membrane fractions of eight eye lenses were transferred into clean glass culture tubes and spiked with deuterated internal standards (1 ng of 7ƃ-hydroxycholesterol d7, 5 ng of 7-Ketocholesterol d7 and 1ng of 5Ŭ,6Ŭ-epoxycholestanol-d7) and vortexed. Membrane fractions were mixed with 2 mL of 2:1 methanol:dichloromethane ((MeOH:DCM) containing 50 µg/ml Butylated hydroxytoluene (BHT) as recommended [66]. The samples were vortexed for 30 s and left to incubate for 30 min at room temperature (RT). Subsequently, 0.67 mL of DCM and 1.2 mL of 0.9% (w/v) KCl were added consecutively, with a 30 sec vortex step after adding each solution. The samples were centrifuged at 1000 *g* for 5 min at RT and the lower lipid phase layer was transferred into a fresh glass tube with a Pasteur pipette. The lipids were dried under $N_2$ gas.

### Liquid chromatography - mass spectrometry

Our samples were analysed based on the methodology developed and published by McDonald and colleagues [66]. The following standards were used and all were purchased from Sigma-Aldrich unless otherwise stated; cholesterol and cholesterol D6: 7-ketocholesterol; 7 -hydroxycholesterol (Sigma); 7 dehydrocholesterol D7 (Cambridge Bioscience) 5, 6 epoxy-cholesterol, 25-hydroxycholesterol D6, desmosterol and desmosterol-D6 using retention times and MRMs for identification of sample peaks [67]. Quantitative analysis was performed with a Shimadzu UHPLC system linked to a hybrid triple-quadrupole mass spectrometer (QTRAP 6500, AB Sciex). For oxysterol analysis, the samples were dissolved in 50 µL of 1:1 acetonitrile:isopropanol (ACN:IPA) and separated on a Kinetex $C_{18}$ HPLC column (150 Ĭ 2.1 mm, 2.6 ɛm particle size; Phenomenex) with mobile phases A (60:40 ACN: $H_2O$, 10 mM $NH_4HCO3$, 0.1% HCOOH) and B (50:50 ACN: IPA, 10 mM $NH_4HCO3$, 0.1% HCOOH). For cholesterol analysis, the samples were dissolved in 500 µL 1:1 ACN:IPA and run under the same conditions on a Cortecs $C_{18}$ UPLC column (100 × 2.1 mm, 1.6 ɛm particle size; Waters). The flow rate was 140 ÕL/ min, and the column was maintained at 60 °C.

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