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Cholesterol Dyshomeostasis And Age Related Macular Degeneration

Bhanu Chandar Dasari

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CHOLESTEROL DYSHOMEOSTASIS AND AGE RELATED MACULAR
DEGENERATION

by

Bhanu Chandar Dasari
Bachelor of Science, Kakatiya University, 1999
Master of Science, University of Pune, 2001

A Dissertation
Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota

May
2012

This dissertation, submitted by Bhanu Chandar Dasari in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Othman Ghribi, Ph.D.

Brij B. Singh, Ph.D.

Colin K. Combs, Ph.D.

James E. Porter, Ph.D.

Saobo Lei, Ph.D.

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.

Wayne E. Swisher, Ph.D.
Dean of the Graduate School

Date

Title Cholesterol Dyshomeostasis and Age Related Macular
 Degeneration

Department Pharmacology, Physiology & Therapeutics

Degree Doctor of Philosophy

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Bhanu Chandar Dasari
April 23, 2012

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ABSTRACT

This dissertation work focused on retinal modifications that are relevant to Age-related macular degeneration (AMD) in cholesterol-fed rabbit model of Alzheimer's disease (AD), as AD and AMD share common features. It is unknown whether cholesterol-fed rabbit model of AD displays any AMD features in retina. Previous research showed 27-hydroxycholesterol (27-OHC) involvement in AD like pathology in organotypic hippocampal slices of rabbit brain and human SHSY-5Y neuroblastoma cells. The extent to which and the mechanisms by which 27-OHC may also cause pathological hallmarks related to AMD are not known. Various studies suggested estrogen's (E2) role in AMD development. 27-OHC is a ligand for estrogen receptor (ER) and liver X receptor (LXR). 25-hydroxycholesterol (25-OHC) and 7-ketocholesterol (7-KC) are also implicated in AMD development. 25-OHC and 7-KC were shown to be ligands of ER and LXR in various cell types. It is unknown whether 27-OHC, 25-OHC and 7-KC influence ER and LXR transcriptional activity in ARPE-19 cells, a spontaneously arising human RPE cell line with normal karyology.

ARPE-19 cells and cholesterol-fed rabbit eyes were used for the study. Paraffin embedded eye cross sections were used for immunohistochemistry. Cholesterol was quantified by cholesterol/cholesteryl ester quantification kit. Oxysterols in the rabbit retinas were measured by mass spectrometry. Western blotting for detecting proteins, CytoTox-ONE homogenous membrane integrity

assay for measuring lactate dehydrogenase from cells, ELISA (Enzyme-linked immunosorbent assay) for quantifying amyloid beta (A β) 1-42 and A β 1-40, tumor necrosis factor α , DCFH-DA (2',7'-dichlorofluorescein-diacetate) assay for measuring reactive oxygen species (ROS), amplex red hydrogen peroxide / peroxidase assay for quantifying hydrogen peroxide and peroxidase activity, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) assay for mitochondrial membrane potential detection, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay for apoptotic cell detection, GSH-Glo assay for glutathione (GSH) quantification, calcium imaging, immunocytochemistry, immunohistochemistry and H&E (hematoxylin and eosin) staining, transfection and dual-luciferase reporter assays were used for this work.

This study showed retinal modifications that are relevant to AMD in cholesterol-fed rabbits. Increased A β levels, decreased apoptosis regulator Bcl-2 levels, increased apoptosis regulator BAX and growth arrest and DNA damage-inducible protein GADD153 proteins, apoptotic cells, and increased generation of ROS were found in retinas from cholesterol-fed rabbit retinas. Furthermore, astrogliosis, drusen-like debris and cholesterol accumulations in retinas from cholesterol-fed rabbits were observed. Oxysterol levels in retinas from cholesterol-fed rabbits were increased. 27-OHC increased A β peptide production, increased caspase 12 and GADD153, reduced mitochondrial membrane potential, triggered Ca²⁺ dyshomeostasis, and increased levels of the nuclear factor NF-kappa-B p65 subunit (NF- κ B p-65) and heme oxygenase 1 (HO-1). Additionally, 27-OHC caused GSH depletion, ROS generation, inflammation and apoptotic-

mediated cell death in ARPE-19 cells. ARPE-19 cells express ER α , ER β , LXR α and LXR β . ER and LXR are transcriptionally active. ER agonist E2 protected cells from 25-OHC and 27-OHC induced cytotoxicity. E2 counteracted 27-OHC and 25-OHC induced mitochondrial membrane potential decline.

Taken together, data demonstrate that cholesterol-enriched diets induce pathological hallmarks suggestive of AMD in rabbit retinas. The cholesterol diet substantially increased concentrations of the major oxysterols, accumulation of which is toxic to retinal cells. Incubation of cells with oxysterols also reproduced the major effects observed *in vivo* with the diet rich in cholesterol. These results strongly suggest that hypercholesterolemia and subsequent increase in oxysterol formation may contribute to the pathogenesis of AMD. Our data also shows that ER modulation may play an important role in the cholesterol and oxysterol effects. Specifically, ER agonists may provide protection against oxysterol deleterious effects on retinas. As well, reduction of cholesterol plasma levels may prevent excess conversion of cholesterol to oxysterols and precludes the incidence of AMD.

CHAPTER I

INTRODUCTION

Eyes are windows to the wonders of the world. The reflected light enters the eye through the cornea, a transparent dome on the front surface of the eye, then travels through the aqueous humor, pupil, lens and vitreous humor to project onto photoreceptors of the retina, whose impulses converge on the optic nerve and then to the brain to be transfigured into image.

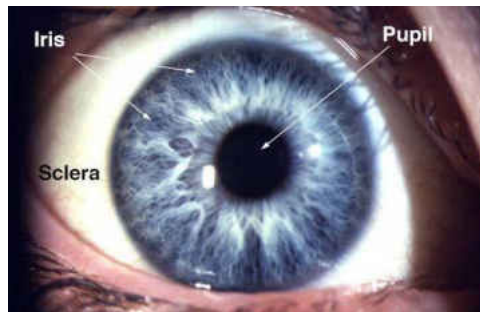


Figure 1: View of the human eye (Webvision, <http://webvision.med.utah.edu/>)

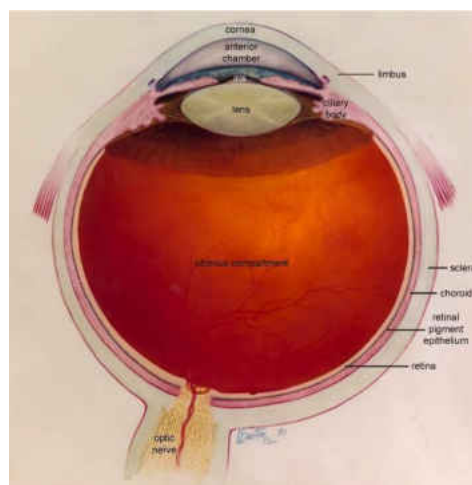


Figure 2: A drawing of eye's cross section (<ftp://ftp.nei.nih.gov/eyean/eye12-72.tif>)

The pupil is central aperture of the iris that allows light to enter the eye (Figure 1). The iris is a colored circular muscle, which controls the size of the pupil so that more or less light, depending on conditions is permitted to enter the eye. Iris color, generally referred as eye color, is due to variable amounts of pigments formed by melanocytes. The sclera also known as white of the eye is the opaque, fibrous, protective outer layer of the eye containing collagen and elastic fiber, which forms part of the supporting wall of the eyeball. The sclera is continuous with the cornea (Figure 2). Furthermore sclera is in continuity with the dura of the central nervous system. The cornea is transparent external surface of the eye that covers both the pupil and the iris. This is the first and most powerful lens of the optical system of the eye and allows, jointly with the crystalline lens, the construction of a sharp image at the retinal photoreceptor level.

The retina is an integral part of the brain

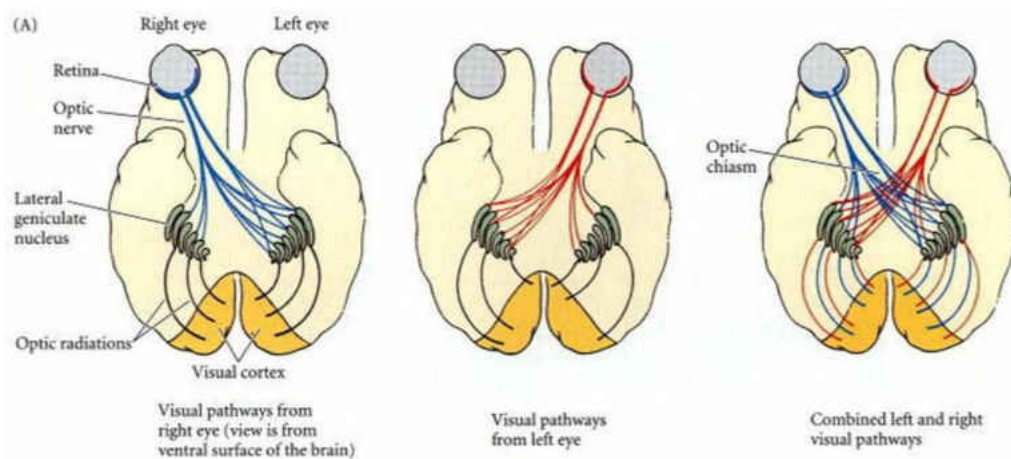


Figure 3: The retina is an integral part of the brain (Gilbert SF. Developmental Biology. 6th edition. Sunderland (MA): Sinauer Associates; 2000. Figure 21.18)

The retina is actually a part of central nervous system (Figure 3). It is formed as a protrusion of the neuroectoderm, a specialized part of the ectoderm, which also develops into the central nervous system (CNS) during embryonic development. The retina is an amazing arrangement of cells (Figure 4) that can translate light into nerve signals, allowing us to perceive the world. The retina comprises intricate neural circuitry that converts the graded electrical activity of photoreceptors into action potentials that travel via axons in the optic nerve to the brain. The macula is an oval-shaped, highly pigmented yellow spot near the center of the retina of human eye. Cones are concentrated in the fovea, which is at the center of macula. The macula is essential for central and near vision, and when the macula deteriorates, persons are impaired in their driving, reading, and other activities of daily living. Because of fewer cell layers, macula is more susceptible to environmental stressors. The progressive damage of the macula is a disease, known as macular degeneration.

The mature retina consists of two distinct tissues, the neural retina and the retinal pigment epithelium (Figure 5). Neural retina consists of five types of neurons, divided into at least 50 subtypes, and glial cells. Five classes of neurons are photoreceptors (PR), bipolar cells (BP), ganglion cells (GC), horizontal cells (HC), and amacrine cells (AC). All cells of the neural retina derive from multipotent progenitor cells. Photoreceptors are capable of phototransduction. There are two types of light-receptor cells in the retina-rods (about a billion) and cones (about 6 million) (Osterberg, 1935). Rods are responsible for our vision in dim light, and cannot discriminate colors. Cones are responsible for color vision, fine details. Both cell types are elongated, narrow, specialized sensory neurons

with two different cellular compartments: the outer segment (OS) contains hundreds of membranous disks, containing rhodopsin, and inner segment contains nucleus and mitochondria. Bipolar cells are situated in the inner nuclear layer and convey information from the outer plexiform cells to the inner retina. They have a cell body from which two sets of processes arise. Dendritic processes of bipolar cells make synapses with photoreceptors and horizontal cells in the outer plexiform layer and their axon terminals contact with amacrine and ganglion cells in the inner plexiform layer. Horizontal cells function in modulating signaling between photoreceptors and bipolar cells. Amacrine cells modulate 70 % of input to retinal ganglion cells (RGCs). Bipolar cells, which are responsible for the other 30 % of input to retinal ganglia, are regulated by amacrine cells. Ganglion cells are found in inner most layer of the retina. Ganglion cells send messages to the brain in the form of action potentials, which are influenced by amacrine cells and bipolar cells.

Glial cells are non-neuronal cells. Generally, the mammalian retina consists of three types of glial cells- microglial cells, astrocytes and Müller (radial glial) cells. Microglial cells are the blood-derived resident immune cells in the retina that have an essential role in host protection against invading microorganisms, launching inflammatory processes, and tissue repair. In species with completely or locally vascularized retinae, astrocytes are also situated in these innermost retinal layers. The Müller cells are the principal glial cells of the vertebrate retina and span the entire retinal thickness (Bringmann, 2006).

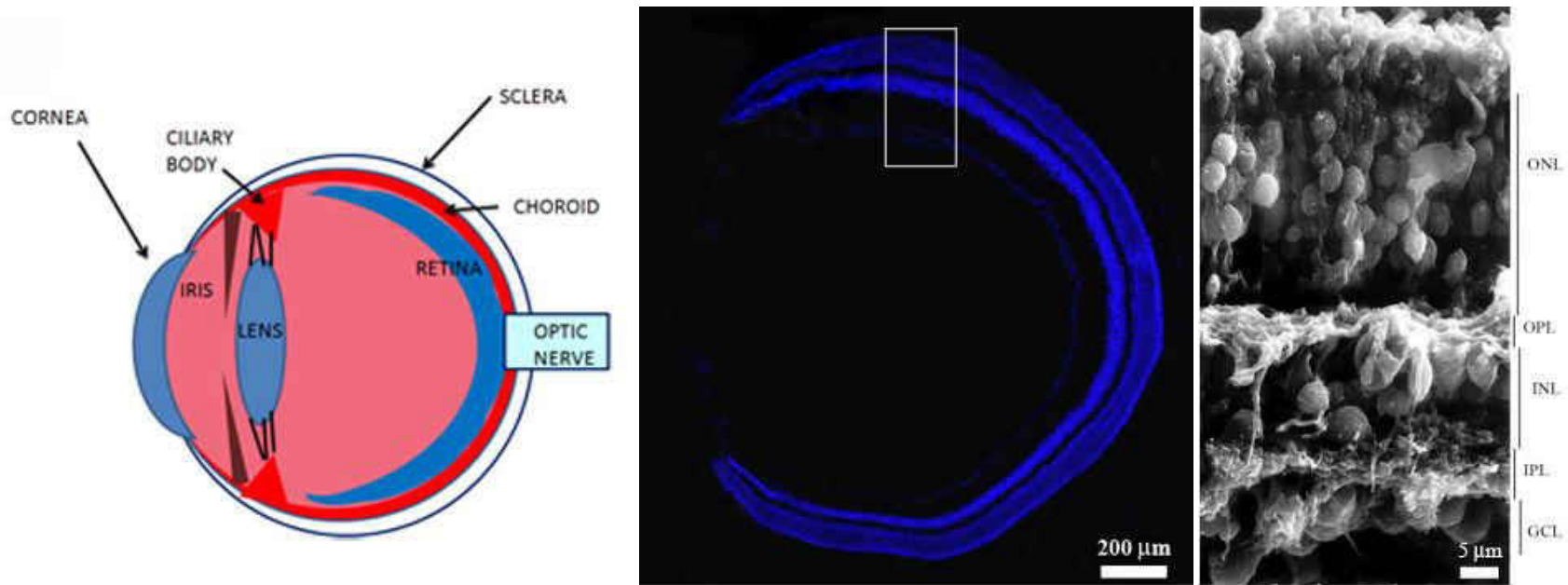


Figure 4: Structure of the eye. A a schematic draw of the eye. B a cross section of the whole retina stained with DAPI (4',6-diamidino-2-phenylindole) to stain the nuclei and viewed under epifluorescence. C a cross retinal section viewed under electron scanning microscope (Microscopy: Science, Technology, Applications and Education A. Méndez-Vilas and J. Díaz (Eds.))

Retinal cells are arranged in precise order, conserved in many species (Cepko, 1993). The mature retina is highly organized structure. The photoreceptor layer (PRL) consists of the inner and outer segments of rod and cone photoreceptors.

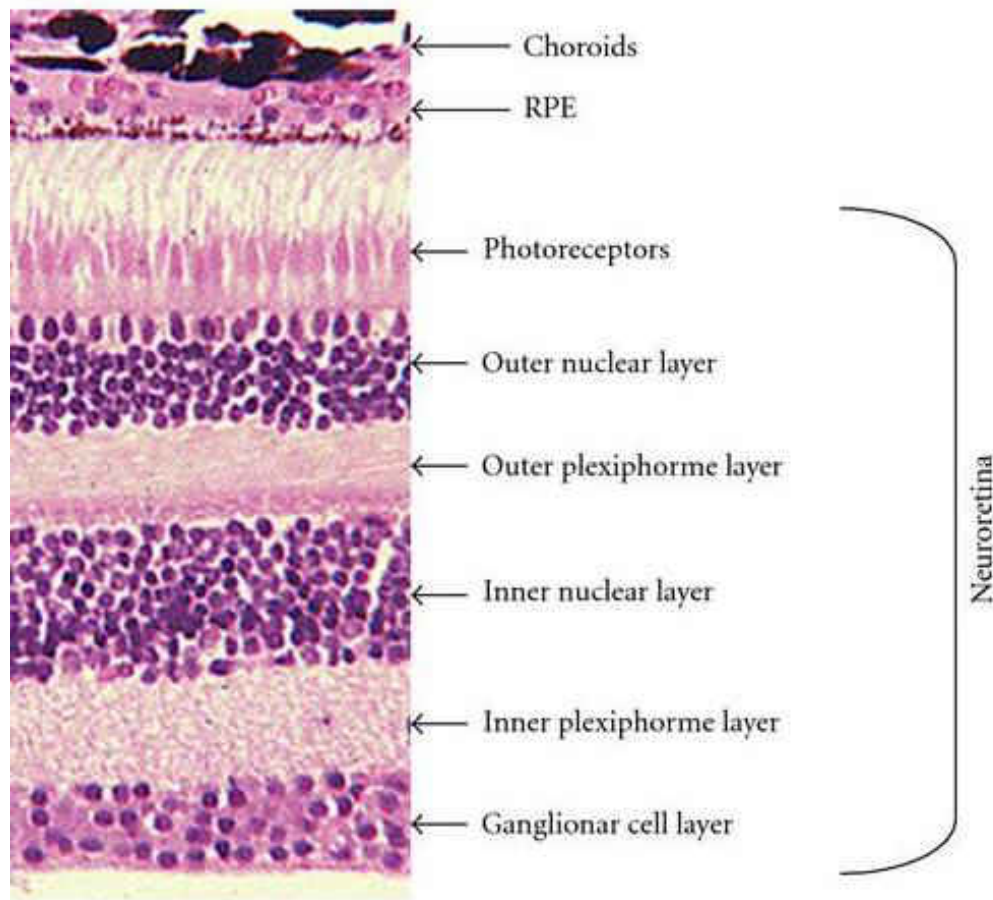


Figure 5: Retinal section showing the location of the retinal pigment epithelium (RPE) (Simo, 2010)

The outer nuclear layer (ONL) is made up of the cell bodies of rod and cone photoreceptors. The outer plexiform layer (OPL) is composed of axons of rod and cones and the dendrites of horizontal and bipolar cells. The inner nuclear layer (INL) contains cell bodies of horizontal, bipolar, amacrine, Müller glia and interplexiform cells. The inner plexiform layer (IPL) contains axons of bipolar

and amacrine cells as well as dendrites of ganglion cells. The ganglion cell layer (GCL), has cell bodies of ganglion cells and displaced amacrine cells and astrocytes. The axons of the ganglion cells form the nerve fiber layer. They pass through the retina, collect in a bundle at the optic disc, and leave the eye to form the optic nerve.

The retinal pigment epithelium (RPE) is the outermost monolayer of tightly connected hexagonal cells between the light sensitive outer segments and the choroid blood supply. RPE plays critical role in the physiology of the underlying photoreceptors which include differentiation and survival of photoreceptors and a crucial target of injury in AMD (Figure 6).

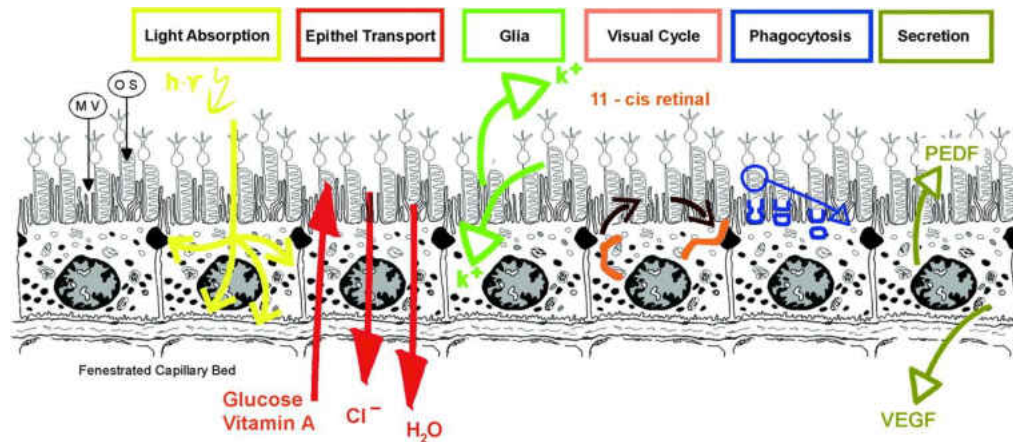


Figure 6: Summary diagram of the major functions of the RPE (Strauss, 2005)

The RPE is essential for development and maintenance of the neural retina (Raymond and Jackson, 1995). RPE participates in the formation of the blood retina barrier and controls the transport of ions and metabolites (Miller and Steinberg, 1979). RPE cells continuously ingest the shed discs of photoreceptor outer segments by phagocytosis. It is believed that a reduced ability to absorb light and to balance for oxidative damage are major factors

initiating the chain of events leading to the onset of A in the older population. RPE degeneration is one of the initial events that occur in AMD (Kopitz, 2004; Nowak, 2006; Young, 1987).

Blood supply to the mammalian retina is carried out by the choroidal blood vessels and the central retinal artery. The choroid receives the most blood flow (65-85 %) (Henkind, 1979) and is vital for the maintenance of the outer retina (particularly the photoreceptors). The remaining 20-30 % flows to the retina through the central retinal artery from the optic nerve head to nurture the inner retinal layers.

Retinal Diseases

The retina is vulnerable to a variety of diseases, including AMD, diabetic retinopathy (DR), retinitis pigmentosa (RP) and other hereditary retinal degenerations, uveitis, retinal detachment, and eye cancers (ocular melanoma and retinoblastoma). The leading reason for visual loss among elderly persons is AMD, which has an increasingly significant social and economic impact in the United States.

AMD is a retinal degenerative disease associated with aging that progressively abolishes sharp, central vision (Figure 7). AMD is a global epidemic with an estimated incidence of 30 to 50 million (Kawasaki, 2010; Nirmalan, 2004; Rein, 2009; Smith, 2001). This disease is characterized by progressive cell damage that targets the choroid, RPE and retina. The burden of AMD to individuals and society is likely to go up as consequence of increased life expectancy and reduced birth rates (Resnikoff, 2004).



Figure 7: Normal vision and the same scene as viewed by a person with age-related macular degeneration (Courtesy: National Eye Institute, National Institutes of Health)

AMD is the most common cause of irreversible vision loss in elderly population (Evans, 2001; Klein, 1998; Mitchell, 1995; VanNewkirk, 2000) and accounts for half of all new cases of registered blindness (Evans and Wormald, 1996). Although AMD is considered as a disease of just the elderly, in fact, even middle-aged individuals are at risk. A 50-year-old American woman is four times more likely to be diagnosed with AMD than breast cancer before reaching 55 (Klein, 1997; Rein, 2009).

Accumulation of extracellular deposits known as drusen (Figure 8), are often associated with pigmentary abnormalities (early AMD). Drusen located between the RPE and Bruch's membrane (BM) is considered as a major hallmark of AMD (Green and Enger, 1993; Sarks, 1980). Early studies suggested and recent studies confirmed that degeneration of the RPE, initiated by membranous debris shed from its basal surface leads to drusen formation (Anderson, 2004; Anderson, 2002; Burns and Feeney-Burns, 1980; Farkas, 1971; Green and Key, III, 2005; Hageman and Mullins, 1999; Ishibashi, 1986a; Kliffen, 1997; Mullins, 2001; Russell, 2000; Sarks, 1976; Sarks, 1999; Sarks, 2007).

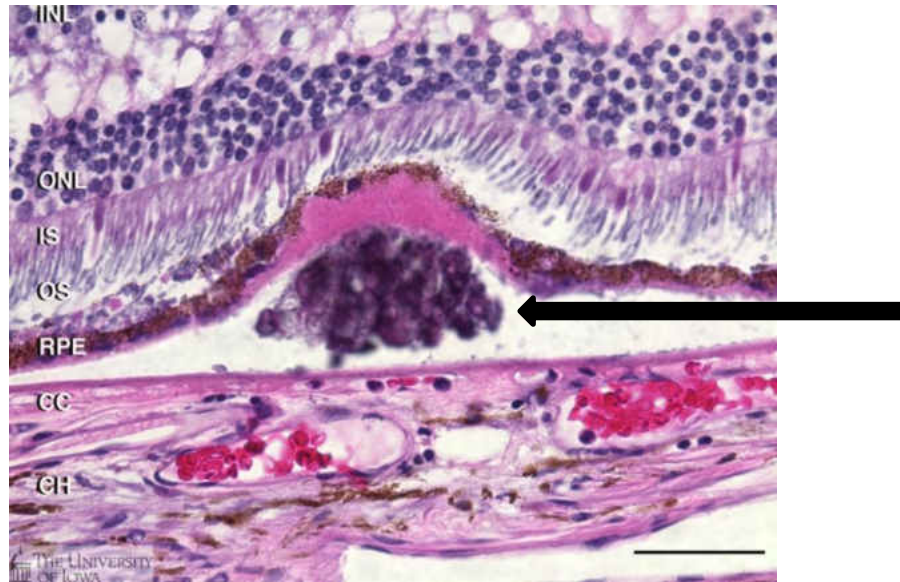


Figure 8: Drusen. H&E stain shows sub-RPE deposition (The University of Iowa Department of Ophthalmology & Visual Sciences; Age-Related Macular Degeneration/Age-Related Maculopathy: A Histopathologic Analysis. Elizabeth A. Faidley, Jessica M. Skeie, and Robert F. Mullins, PhD)

Drusen may raise the risk of developing AMD (Green, 1999). Drusen are composed of acute phase proteins, complement components, apolipoproteins, lipids, polysaccharides, cholesterol in unesterified and esterified forms along with various other molecules (An, 2006; Curcio, 2005a; Curcio, 2005b; Li, 2007; Malek, 2003; Rudolf and Curcio, 2009). A β accumulation has also been demonstrated to be associated with drusen in eyes from AMD patients (Dentchev, 2003; Johnson, 2002; Luibl, 2006), mice models for AMD (Malek, 2005) and in RPE cells (Yoshida, 2005). A β immunization may be a pertinent therapeutic approach for both AD and AMD (Ding, 2008; Ding, 2011).

Advanced AMD includes geographic atrophy (dry AMD) and choroidal neovascularization (wet AMD) (Figure 10). Patients complain of distorted vision (metamorphopsia), inability to perceive or discern colors (discromatopsia), lost

vision, an area of depressed vision corresponding with the point of fixation and interfering with central vision (central scotoma). Geographic atrophy is a common type of late AMD in the European population, whereas exudative AMD is a major type of late AMD in the Asian population.

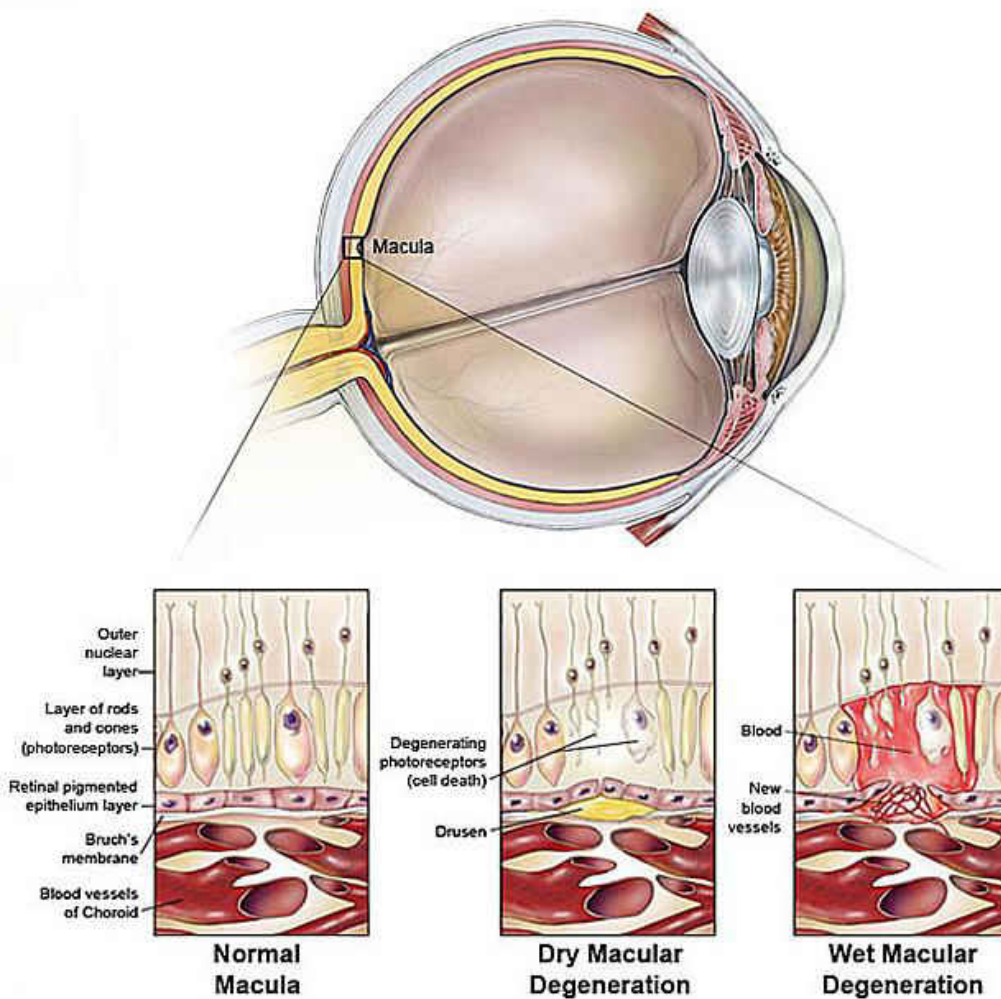


Figure 9: The normal macula compared to wet and dry macular degeneration (Illustration by Bob Morreale, provided by courtesy of the American Health Assistance Foundation <http://www.ahaf.org/macular/about/understanding/normal-macula-compared.html>)

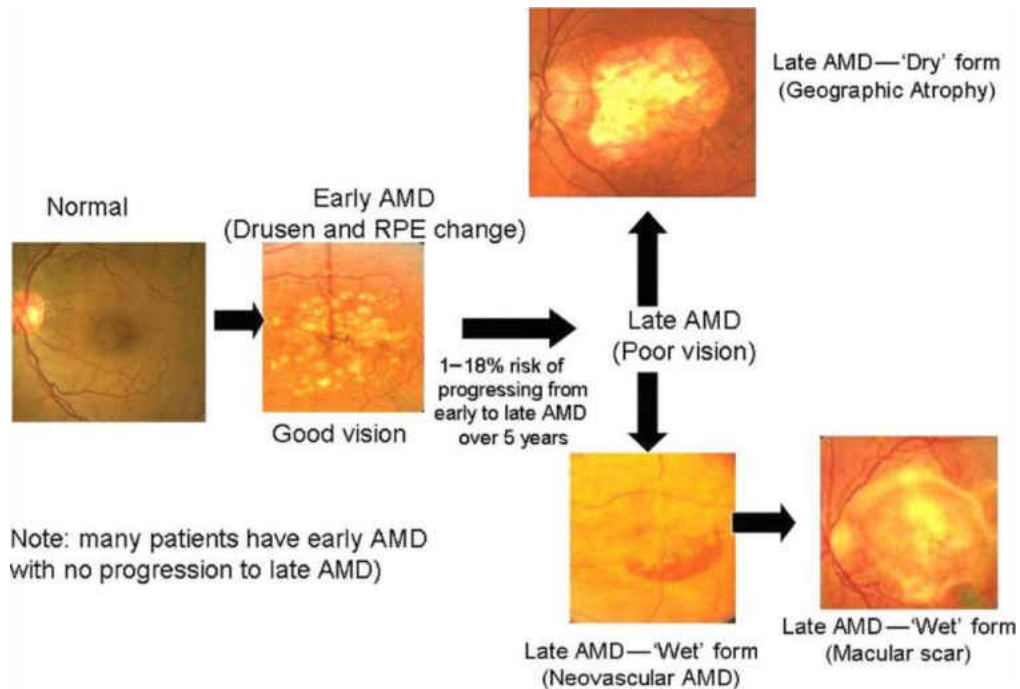


Figure 10: Classification of age-related macular degeneration. (Cook H L Br Med Bull 2008; 85:127-149)

Presently, there are no effective treatments for early AMD. Treatments for late-stage disease are limited to photodynamic therapy with benzoporphyrin (verteporfin, Visudyne; Novartis, Basel, Switzerland) (Treatment of Age-related Macular Degeneration With Photodynamic Therapy Study Group, 1999), macular translocation, and antivascular endothelial growth factor drugs such as pegaptanib (Macugen; Pfizer, New York, NY),(Gragoudas, 2004). An aptamer targeting vascular endothelial growth factor (VEGF)-A; ranibizumab (Lucentis; Genentech, South San Francisco, CA), an anti-VEGF-A antibody Fab fragment; bevacizumab (Avastin; Genentech), a full-length anti-VEGF-A antibody, have essentially changed the clinical management of choroidal neovascularization (CNV), as these drugs were the first to improve vision (Brown, 2006; Gehrs,

2006; Gragoudas, 2004; Hooper and Guymer, 2003; Mordenti, 1999; Rosenfeld, 2006; Tufail, 2010).

AMD's social and economic factors, unclear pathogenesis and limited existing therapies, has driven researchers to carry out studies designed to identify risk factors.

Large epidemiologic studies investigating risk for AMD (Connell, 2009)

- Beaver Dam Eye Study, USA (1988–2005), Cross-Sectional, Prospective (Klein, 2001; Klein, 1997; Klein, 1999; Klein, 2002; Klein, 2007; LeClaire, 1998)
- Blue Mountains Eye Study, Australia (1992–2004), Cross-Sectional, Prospective (Mitchell, 1995; Mitchell, 2002; Tomany, 2004)
- Rotterdam Study, Holland (1990–2004), Cross-Sectional, Prospective (Klaver, 2001; Smith, 2001; Tomany, 2004; van Leeuwen, 2003)
- Pathologies Oculaires Liees a L'Age, France (1995–2000), Cross-Sectional, Prospective (Delcourt, 1999; Delcourt, 2005)
- Los Angeles Latino Eye Study, USA (2000–2003), Cross-Sectional (Varma, 2004)
- Melbourne Visual Impairment Project, Australia (1992-1999), Cross-Sectional, Prospective (McCarty, 2001; Mukesh, 2004)
- Reykjavik Eye Study, Iceland (1996–2001), Cross-Sectional, Prospective (Arnarsson, 2006; Jonasson, 2003; Jonasson, 2005)
- Beijing Eye Study, China (2001), Cross-Sectional, (Klein, 2001; Xu, 2006)

- Copenhagen City Eye Study, Denmark (1986–2002), Cross-Sectional, Prospective (Buch, 2001; Buch, 2005a; Buch, 2005b)
- Andhra Pradesh Eye Disease Study, India (1996–2000), Cross-Sectional (Krishnaiah, 2005)
- Barbados Eye Studies Barbados (1987–2003), Cross-Sectional, Prospective (Hyman, 2001)
- Salisbury Eye Evaluation Project, USA (1993), Cross-Sectional, (Bressler, 1989)
- Proyecto VER, USA (1997–1999), Cross-Sectional (Munoz, 2005)
- Baltimore Eye Survey, USA (1985–1988), Cross-Sectional (Friedman, 2004)
- Aravind Comprehensive Eye Survey, India (1995–1997), Cross-Sectional (Nirmalan, 2004)
- European Eye Study, 7 European Countries (2000–2003), Cross-Sectional (Augood, 2006) (Chakravarthy, 2007)
- Hisayama Study, Japan (1998–2003) Cross-Sectional, Prospective (Miyazaki, 2003; Miyazaki, 2005)
- Framingham Eye Study, USA (1973–1975), Cross-Sectional (Kahn, 1977a; Kahn, 1977b)
- National Health and Nutrition Examination Survey I, USA (1971–1972), Cross-Sectional (Goldberg, 1988)
- National Health and Nutrition Examination Survey III, USA (1988–1994), Cross-Sectional (Heuberger, 2001; Heuberger, 2002)

- Cardiovascular Health Study USA, (1997–1998), Cross-Sectional (Klein, 2003a; Robman, 2004)
- Atherosclerosis Risk in Communities Study, USA (1993–1995), Cross-Sectional (Cheung, 2007; Grosso, 2007)
- MRC Trial of Assessment and Management of Older People in the Community, UK (1996–2000), Cross-Sectional (Evans, 2001)

Risk factors examined in the epidemiologic studies

Genetic predisposition

- Family history of age related maculopathy (ARM), Complement Factor H gene, Apolipoprotein E gene, LOC gene

Cardiovascular disease

- Clinical Evidence of Atherosclerosis
 - Angina/Heart attack/Stroke
- Subclinical evidence of atherosclerosis
 - Carotid atherosclerosis, Aortic atherosclerosis
- Cigarette smoking
- Diabetes mellitus
- Hypertension and associated disease
- Ischemic cerebral white matter changes, Abnormalities of the retinal vasculature
- Cholesterol
 - Total cholesterol, Low-density Lipoprotein (LDL) cholesterol, High-density Lipoprotein (HDL) cholesterol

- Obesity
- Female sex hormones
 - Endogenous estrogen exposure
 - Age at menarche, Age at menopause, Number of pregnancies
 - Exogenous estrogen exposure
 - Oral contraceptives, Hormone replacement therapy
- Novel risk factors for atherosclerosis
 - Lipid-related Factors
 - Apolipoproteins, Lipoproteins
 - Inflammatory markers
 - C-reactive protein, Interleukins, Serum Amyloid A, Vascular and Cellular Adhesion Molecules, White Blood Cell Count
 - Homocysteine/Folate/Vitamin B12/Vitamin B6
 - Infectious agents
 - Cytomegalovirus, Helicobacter pylori, Chlamydia pneumonia

Indicators of Inflammation

- Systemic Diseases with Inflammatory Components
 - Gout, Emphysema
- Anti-inflammatory Medications
 - Nonsteroidal anti-inflammatory drugs (NSAIDs), Steroids
- Markers of Systemic Inflammation
 - White Blood Cell Count, Serum Albumin, Plasma Fibrinogen, C-reactive protein (CRP), Complement Factor H Y402H Polymorphism, CRP Haplotype, Serum Amyloid A, Interleukin-6,

TNF- α

Markers of Endothelial Dysfunction

- Intercellular Adhesion Molecule-1, E-Selectin

Indicators of Oxidative Stress

- Anti-oxidants
 - Vitamin C, Vitamin E, Vitamin A,
 - Carotenoids
 - Lutein, Zeaxanthin, α - and β -Carotene, β -Cryptoxanthin, Lycopene
 - Enzymes
 - Plasma Glutathione Peroxidase, Superoxide Dismutase
 - Trace Elements
 - Zinc
- Pro-oxidant status
 - Dietary fat intake
 - Total fat, Saturated fat, Polyunsaturated fat, Fish/Fish oils
 - Visible Light Exposure
 - Sunlight, Ultraviolet-B

Ocular Factors

- Refractive Error
 - Emmetropia, Myopia, Hypermetropia
- Iris Color
- Cataract

- Nuclear Sclerosis, Cortical Lens Opacities, Posterior Subcapsular
Cataracts

- Cataract Surgery

Miscellaneous Factors

- Alcohol Consumption
 - Beer, Wine, Spirits
- Medication Use
 - E2, Lipid-lowering agents, CNS medications, NSAIDs, Anti-hypertensive medications
- Coffee Consumption
- Frailty
- Physical Activity

This study concentrated on one of the above mentioned risk factor which is cholesterol. The pathogenesis of AMD is still not clarified completely. Among these factors, cholesterol metabolism is believed to contribute since cholesterol accumulation in Bruch's membrane is implicated in the etiology and progression of AMD (Chen, 2010; Curcio, 2005a; Curcio and Millican, 1999; Curcio, 2005b; Klein, 2003b; Neale, 2010).

Cholesterol, oxysterols, and Alzheimer's disease

Cholesterol is an essential component of cellular membranes of most vertebrates. It is essential for various cellular functions, including the maintenance of appropriate membrane permeability and fluidity, as well as the regulation of integral membrane protein function (Maxfield and Tabas, 2005; Xu and London,

2000). Cholesterol homeostasis is maintained by the interplay between synthesis, uptake, and degradation.

Oxysterols are oxygenated derivatives of cholesterol. Because excess free cholesterol is toxic to the cell, maintaining narrow limits of cellular cholesterol levels is crucial for cell health (Feng, 2003; Tabas, 2002). Cellular cholesterol homeostasis is sensitive to the level of cholesterol and to oxygenated derivatives of cholesterol, termed oxysterols.

Alzheimer's disease (AD) is one form of dementia that gradually gets worse over time. It affects memory, thinking, and behavior. Various studies including epidemiological studies showed cholesterol and AD link (Ghribi, 2008; Kivipelto and Solomon, 2006; Sparks, 1994; Wolozin, 2004). Studies show that increased plasma cholesterol levels are associated with atherosclerosis and increased risk of dementia, including AD (Kivipelto and Solomon, 2006; Solomon, 2009). Accumulations of A β and tau proteins are characteristic of the disease.

Cholesterol-fed rabbit as a model of AD

Rabbits have been used in atherosclerosis studies because they exhibit hypercholesterolemia quickly upon administration of a high-cholesterol diet. They are also highly responsive to inducement of atherosclerotic lesions similar to human atherosclerosis (Finking and Hanke, 1997; Yanni, 2004). Whereas the first indication of association between cholesterol and the accumulation of A β plaques in the brain came from studies on rabbits fed with 2 % cholesterol- diet for 4, 6, and 8 weeks. These rabbits showed mild to moderate to severe

accumulation of intracellular immunolabelled β -amyloid (Sparks, 1994). Our laboratory has been using cholesterol-fed rabbit as a model for studying AD.

Visual problems in AD

There is increasing evidence of a link between AD and retinal diseases including glaucoma and AMD, as evidenced by the deposition of $A\beta$ peptide in both diseases (Guo, 2010). Visual problems have been observed even in the initial stages of AD (Katz and Rimmer, 1989; Sadun, 1987). Reduction in the number of ganglion cells and in the thickness of the nerve-fiber layer has been observed in AD patients (Hinton, 1986). Eyes, in particular retina is affected in AD (Berisha, 2007; Iseri, 2006; Ning, 2008; Paquet, 2007; Parisi, 2001; Parisi, 2003; Shimazawa, 2008).

AD and AMD share common pathology

Intriguingly, AMD has many pathological features that are common to AD, including the deposition of $A\beta$ peptide (Anderson, 2004). $A\beta$ levels are regulated by generation from amyloid precursor protein (APP) upon initial cleavage by beta-secretase 1 (BACE-1) and degradation by enzymes that include insulin-degrading enzyme (IDE). $A\beta$ is suggested to play a key role in AD pathogenesis by triggering oxidative stress, inflammation and cell death (Querfurth and LaFerla, 2010). $A\beta$ accumulation has also been demonstrated to be associated with drusen in eyes from AMD patients (Dentchev, 2003; Johnson, 2002; Luibl, 2006), mice models for AMD (Malek, 2005) and in RPE cells (Yoshida, 2005). In addition to drusen deposits, oxidative stress, apoptosis and accumulation of $A\beta$ peptide are also hallmarks of AMD (Ding, 2009). Interestingly, these hallmarks are also characteristics of AD (Butterfield, 2002; Kaarniranta, 2011). Furthermore, $A\beta$

accumulation is the leading neuropathological change that correlates with the diagnosis of AD, and is considered a key player in the pathogenesis of AD by inducing oxidative stress and apoptotic cell death. The causes of AMD and AD are not well defined, but several factors including diet, environment, and genetic susceptibility likely contribute to the pathogenesis of these diseases (Ohno-Matsui, 2011)

Cholesterol, oxysterols and AMD

Hypercholesterolemia is one risk factor among others for developing AMD (van Leeuwen, 2003). Similar to AD, the causes of AMD are not fully understood. The retina synthesizes cholesterol endogenously and also receives from blood circulation (Fliesler, 1993; Fliesler and Keller, 1997; Tserentsoodol, 2006b). The role of cholesterol metabolism in the pathogenesis of AMD has also been raised (Johnson, 2002; Mares-Perlman, 1995). Cholesterol (free and esterified) is highly distributed in the macular drusen in humans (Curcio, 2005b; Li, 2007; Rudolf and Curcio, 2009). The source of the cholesterol that accumulates in the retina is suggested to derive from both local cells and plasma origins (Curcio, 2001; Holz, 1994; Malek, 2003; Trivino, 2006). The relative contributions of LDL-derived cholesterol and endogenously synthesized cholesterol to total retinal cholesterol are unknown.

Epidemiological and animal studies have suggested a link between high plasma cholesterol levels and AD (Solomon, 2009). As well, high intake of cholesterol and saturated fat have long been suspected to increase the risk for AMD (Mares-Perlman, 1995). Because of the putative links between cholesterol and AD (Bjorkhem, 2006) as well as cholesterol and AMD (van, 2004),

cholesterol homeostasis in the brain and retina is now drawing more attention. Although, there is no consensus on the association of plasma lipid levels and AMD, two recent genome wide association studies implicated cholesterol metabolism involvement in AMD (Chen, 2010; Neale, 2010). In AMD, drusen accumulation on Bruch's membrane exhibit increased deposition of lipids, including cholesterol. Drusen contains histochemically detectable lipid (Curcio, 2001; Curcio, 2005b; Haimovici, 2001; Wang, 2010; Wolter and Falls, 1962) including cholesterol in 2 chemical forms, unesterified (UC) and esterified to a long chain fatty acid (EC) (Curcio, 2005a). Various laboratories showed changes in intraretinal and epiretinal capillary endothelial cells, an increase in lipids, ultrastructural changes similar to AMD in the retinas of cholesterol-fed rabbits (Miceli, 2000; Ong, 2001; Ramirez, 2006; Salazar, 2007; Trivino, 2006). In one study where authors substituted the hyperlipemic diet with standard one normalized the blood-lipid levels and decreased ocular lipid build-up. However, ocular damage regression was partial (Ramirez, 2006).

Oxysterols are oxygenated derivatives of cholesterol that have diverse physiological and biochemical functions like cholesterol homeostasis, cell differentiation, apoptosis, modulation of vesicular movement, regulation of nuclear receptors and calcium transport and also involved in many diseases because abnormal oxysterol levels can cause oxidative stress, inflammation and apoptotic cell death (Ares, 2000; Bjorkhem, 2002; Chen, 2002; Fang, 1996; Hanley, 2000; Hayden, 2002; Javitt and Javitt, 2009; Joffre, 2007;

Studies investigating the relationship between cholesterol and AMD

Study	No. of cases	Measure of risk factor	Type of ARM	Odds ratio or relative risk	95 % confidence interval
Cross-Sectional Studies					
Cardiovascular Health Study (Robman, 2004)	2361	Serum total cholesterol (per 10 mg/dL increase)	ARM	0.95	0.91–0.98
National Health and Nutrition Examination Survey III (Goldberg, 1988)	8270	HDL cholesterol (per mmol/L)	Early ARM	1.30	0.99–1.71
Pathologies Oculaires Liees a L'Age (Delcourt, 2001)	2584	Cholesterol (per mmol/L)	Soft drusen	0.88	0.79–0.99
			Late ARM	0.97	0.71–1.31
Blue Mountains Eye Study (Smith,	3654	HDL Cholesterol (per mmol/L)	Soft drusen	1.07	0.97–1.17
			Late ARM	0.97	0.71–1.31
Blue Mountains Eye Study (Smith,	3654	Cholesterol (per 10 mg/dL)	Early ARM	0.95	0.84–1.09

Study	No. of cases	Measure of risk factor	Type of ARM	Odds ratio or relative risk	95 % confidence interval
2001)			Late ARM	1.08	0.92–1.27
Prospective studies					
Beaver Dam Eye Study (Klein, 2007)	3684	Serum Cholesterol (per 10 mg/dL increase)	Geographic Atrophy	1.29	1.05–1.58
Rotterdam Eye Study (van Leeuwen, 2003; van Leeuwen, 2004)	4776	Serum Cholesterol (per 10 mg/dL)	Early ARM	0.96	0.84–1.09
		HDL Cholesterol (per mmol/L)	Early ARM	1.38	0.92–1.79

Ares, 2000; Bjorkhem, 2002; Chen, 2002; Fang, 1996; Hanley, 2000; Hayden, 2002; Javitt and Javitt, 2009; Joffre, 2007; Komuves, 2002; Ong, 2003; Panini and Sinensky, 2001; Riendeau and Garenc, 2009; Smith and Johnson, 1989; Vejux, 2008).

Oxysterols result from either autoxidation or enzymatic oxidation of cholesterol (Figure 11). While 7-KC is the major oxysterol generated by autoxidation on the B hydrocarbon ring of cholesterol, 24-OHC, 25-OHC and 27-OHC are major oxysterols produced by enzymatic oxidation on the lateral chain of the cholesterol structure. Oxysterols have been implicated in the process of aging and degenerative diseases of the human eye (Girao, 1998; Lutjohann, 2000; van den Kommer, 2009). It was demonstrated that cholesterol oxides are toxic to primary cell cultures obtained from rat neuroretinas (Chang and Liu, 1998). Oxysterols, 25-OHC and 7-KC are shown to induce oxidative stress, apoptosis and are cytotoxic to R28 neuroretinal and ARPE-19 retinal pigment epithelium cell lines (Ong, 2003).

Several studies started revealing some aspects of oxysterol formation and function in retina and RPE (Joffre, 2007; Moreira, 2009; Rodriguez and Fliesler, 2009). Currently, the mechanisms by which cholesterol may increase the incidence of AMD are not clear. Several lines of evidence suggest that oxidized cholesterol metabolites (oxysterols) may be the link by which cholesterol contributes to the pathogenesis of AMD. The oxysterol pathway has been proposed as a unifying hypothesis for the cause of AMD (Javitt, 2007; Javitt, 2008; Javitt and Javitt, 2009). 25-OHC and 7-KC are major oxysterols in oxidized LDL (oxLDL) (Colles, 2001). Presence of 7-KC in lipid deposits from

primate retina suggested oxysterol involvement in AMD development (Moreira, 2009). 7-KC treated ARPE-19 showed some of the characteristics of apoptosis (Luthra, 2006).

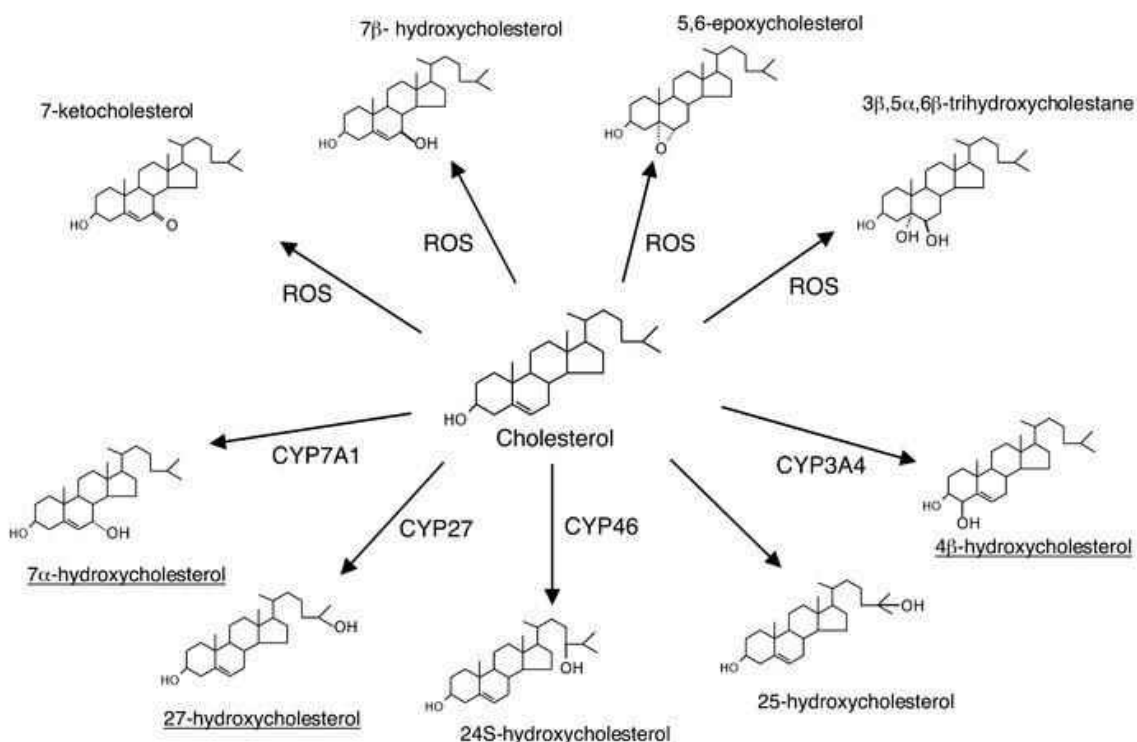


Figure 11: Cholesterol metabolites. Primary cholesterol oxygenation reactions mediated by different cytochrome P-450 species or occurring nonenzymatically in the presence of reactive oxygen species (Bjorkhem, 2002)

Cytotoxicity of oxLDL on RPE cells was shown to be dependent on 7-KC formation (Rodriguez, 2004). 7-KC and 25-OHC showed to have cytotoxic, pro-oxidative, and/or angiogenic activities on ARPE-19 cells (Dugas, 2010).

Oxysterols, Estrogen Receptor, and Liver X Receptor

27-OHC has been demonstrated to bind to both ER and LXR (Fu, 2001; Kim, 2009; Song and Liao, 2000; Umetani, 2007). 27-OHC is the first identified

endogenous selective ER modulator that has agonist activity in breast cancer cells and antagonistic activity in cardiovascular system (DuSell, 2008; Umetani, 2007). 27-OHC is shown to be an endogenous ligand for LXR in cholesterol-loaded cells (Fu, 2001). 25-OHC shown to inhibit E2 activation of ER, whereas also elicits estrogenic effects in cancer cells and cardiomyocytes (Lappano, 2011). 25-OHC is revealed to be natural endogenous ligand for LXRs (Chen, 2007; Janowski, 1996). Inability of 7-KC to antagonize ERs, and E2 dependent nitric oxide synthase (NOS) activity was also shown (Umetani, 2007). 7-KC has been described as an LXR agonist (Janowski, 1999). 7-KC mediated induction of VEGF was attenuated by an LXR antagonist cholesterol sulfate, suggesting LXR involvement (Moreira, 2009).

Dissertation work

Previous studies in our laboratory showed that cholesterol-enriched diets increase A β levels, oxidative stress and cell death in rabbit brains. Since cholesterol enriched diet leads to AD like pathology and retinal degeneration is widely witnessed in AD, retinal modifications in cholesterol-fed rabbits were investigated.

Our laboratory and others suggested oxysterols may be the link between high cholesterol diet and AD like pathology in these cholesterol-fed rabbits. 27-OHC induces AD-like pathology by increasing A β production and triggering apoptotic cell death in human neuroblastoma SH-SY5Y cells (Prasanthi, 2009; Rantham Prabhakara, 2008) and in organotypic slices from rabbit hippocampus (Ghribi, 2009; Sharma, 2008).

As 27-OHC shown to cause AD-like pathology by increasing A β production and triggering apoptotic cell death in human neuroblastoma SH-SY5Y cells and in organotypic slices from rabbit hippocampus, this dissertation work focused on influence of 27-OHC on pathological hallmarks that are common to both AMD and AD by using the human retinal pigment epithelial cell line, ARPE-19.

Various studies suggested E2 may play an important role in AMD development. 27-OHC is the first identified endogenous selective estrogen receptor modulator. LXR, to which 27-OHC is a ligand, is also implicated in AMD development. 25-OHC and 7-KC are also implicated in AMD development. 25-OHC and 7-KC were shown to be ligands of ER and LXR in various cell types.

The existence of ER α , ER β , LXR α and LXR β in ARPE-19 cells was investigated. ER and LXR functional activity studied using dual luciferase reporter assays. 27-OHC, 25-OHC and 7-KC influence on ER-responsive luciferase activity and LXR-responsive luciferase activity was evaluated. Potential beneficial or detrimental effects of ER agonist E2, antagonist ICI182780 (ICI), LXR agonist GW3965 (GW), and antagonist 5 α -6 α -epoxycholesterol-3-sulfate (ECHS) in presence of 27-OHC, 25-OHC and 7-KC were examined.

CHAPTER II

METHODS

This chapter lists all chemicals, methods, and instruments for all cell culture experiments and animal experiments that were used in the dissertation.

Material	Company
25-hydroxycholesterol	Steraloids, Wilton, NH
27-hydroxycholesterol	Medical Isotopes Inc, Pelham, NH
7-ketocholesterol	Sigma Aldrich, St Louis, MO
2-Mercaptoethanol	Bio-Rad, Hercules, CA
35 mm glass bottom culture dishes	MatTek Corp, Ashland, MA
5 α -6 α -epoxycholesterol-3-sulfate	Steraloids, Wilton, NH
75 cm ² flasks, 6, 96-well plates	Corning, Corning, N. Y
Alcoholic Z-Fixative	Excalibur Pathology, Moore, OK, USA
Ammonium persulfate	Amresco, Solon, OH
Amplex Red Hydrogen Peroxide/ Peroxidase assay	Invitrogen, Carlsbad, CA
ARPE-19 cell line	ATCC, Manassas, VA
A β 1-42 and A β 1-40 ELISA	Invitrogen, Carlsbad, CA
BCA assay	Pierce Chemical Co, Rockford, IL
Bioimaging System	Ultra Violet Products, San Gabriel, CA

CCD imaging system	Compix Inc, Cranberry, PA
Cell counter	Invitrogen, Carlsbad, CA
Centrifuge	IEC Multi RF, Thermo Electron Corporation, Waltham, MA
Cholesterol/cholesteryl ester quantification kit	BioVision, Mountain View, CA
Chow	Harlan Teklad Global Diets, Madison, WI
Confocal system	Zeiss, Thornwood, NY, USA
Cyto Tox-ONE Homogenous membrane integrity assay kit	Promega, Madison, WI
Dichlorofluorescein diacetate	Sigma Aldrich, St Louis, MO
DMEM/F12 Glutamax media	Gibco Laboratories, Grand Island, NY
DMSO	Sigma Aldrich, St Louis, MO
Dounce glass homogenizer	Wheaton, Millville, NJ
Dual luciferase assay	Promega, Madison, WI
Dulbecco's PBS	Invitrogen, Carlsbad, CA
Enhanced chemiluminescence Western blotting substrate	Bio-Rad, Hercules, CA
ER reporter, LXR reporter	SABioscience, Frederick, MD, USA
Ethanol 200 proof, anhydrous	Sigma Aldrich, St Louis, MO
FBS	Atlanta Biologicals, Norcross, GA
Fura-2AM	Calbiochem, La Jolla, CA
Gel electrophoresis system	Bio-Rad, Hercules, CA
Glycine	Bio-Rad, Hercules, CA
GSH- Glo assay Kit	Promega, Madison, WI
GW3965	Sigma Aldrich, St Louis, MO

CO ₂ incubator	Shell Lab, Sheldon Manufacturing, USA
JC-1 assay	Biotium, Hayward, CA
Laemmli sample buffer	Bio-Rad, Hercules, CA
Laminar flow cabinet	Labgard classII, typeA2, NuAire, Inc., Plymouth, MN
Lipofectamine LTX and PLUS	Invitrogen, Carlsbad, CA
MPER	Promega, Madison, WI
Methanol	BDH Chemicals, Poole, England
Microplate reader	Molecular Devices, Menlo Park, CA
Microscope	Zeiss Invertoskop 40C, Carl Zeiss, Thornwood, NY
Milk	Bio-Rad, Hercules, CA
Ms TNF- α ELISA	Invitrogen, Carlsbad, CA
NE-PER extraction kit	Thermo Scientific, Rockford, IL
NEXT GEL Solution	Amresco, Solon, OH
Opti-MEM serum free media	Invitrogen, Carlsbad, CA
Orka imaging camera	Hamamatsu Photonics, Hamamatsu, Japan
PCI software	Compix Inc, Cranberry, PA
Paraffin	McCormick Scientific, St. Louis, MO
Penicillin, Streptomycin	Sigma Aldrich, St Louis, MO
pH meter	SympHony, VWR Scientific Products, USA
PowerPac Basic Power Supply	Bio-Rad, Hercules, CA
Protease and phosphatase	Thermo Scientific, Rockford, IL
PVDF membrane	Bio-Rad, Hercules, CA
Running buffer for SDS-PAGE	Amresco, Solon, OH

TEMED	Bio-Rad, Hercules, CA
Tris	Bio-Rad, Hercules, CA
Trypsin	Invitrogen, Carlsbad, CA
T-PER extraction kit	Thermo Scientific, Rockford, IL
TUNEL assay	Promega, Madison, WI

ARPE-19 cell culture

ARPE-19 (human retinal pigment epithelial cells – ATCC-CRL-2302) are spontaneously arising retinal pigment epithelial cells derived from the normal eyes of a 19-year-old male who died from head trauma in a motor vehicle accident. It was established in 1986 by Amy Aotaki-Keen and is the only human RPE cell line available for research. These cells form stable monolayer, which display morphological and functional polarity. ARPE-19 cells express the RPE-specific markers cellular retinaldehyde-binding protein (CRALBP) and retinal pigment epithelium-specific 65 kDa protein (RPE-65) (Dunn, 1996). Cells were grown in Dulbecco's modified eagle medium: Nutrient mixture F-12 (DMEM/F-12) glutamax media with 10 % fetal bovine serum (FBS) and standard antibiotics (100 IU/mL penicillin, and 100 µg/mL streptomycin in a 5 % CO₂, 37 °C incubator. Media containing FBS and antibiotics was changed twice weekly. Cell cultures were routinely subcultivated in the ratio of 1:3 by trypsinization followed by phosphate buffered saline (PBS) wash (NaCl-137 mmol/L, KCl-2.7 mmol/L, Na₂HPO₄ 2 H₂O-10 mmol/L, KH₂PO₄-2.0mmol/L; pH-7.4). Complete growth media was added to detached cells to inactivate trypsin. After centrifuging at 125 x g for 10minutes, the cells were suspended in fresh complete growth media and

were transferred to 75 cm² flasks for propagating, 6-well plates or 96-well plates for various assays.

ARPE-19 cell culture treatments

Stock solutions of 27-OHC, 25-OHC and 7-KC were prepared in 100 % ethanol or dimethyl sulfoxide (DMSO) and stored at -80 °C. Working concentrations were prepared by dissolving stock solutions in complete growth media. When cells reached confluency, they were treated with 0, 10 or 25 µM 27-OHC in culture media at 37 °C for 24 h. The concentrations of the 27-OHC used in the present study are the same as those demonstrated to cause AD-like pathology in human neuroblastoma cells and in organotypic slices (Prasanthi, 2009; Sharma, 2008). Joffre and colleagues have used concentrations up to 50 µM of 7-KC, 24-OHC, and 25-OHC (Joffre, 2007). For experiments involving ER and LXR ligands cells were treated with 10 µM 25-OHC, 10 µM 25-OHC+10nM E2, 10 µM 25-OHC+1 µM ICI182780, 10 µM 25-OHC+1 µM GW3965, 10 µM 25-OHC+1 µM ECHS; 10 µM 27-OHC, 10 µM 27-OHC+10nM E2, 10 µM 27-OHC+1 µM ICI 182780, 10 µM 27-OHC+1 µM GW3965, 10 µM 27-OHC+1 µM ECHS; 25 µM 7-KC, 25 µM 7-KC+10nM E2, 25 µM 7-KC+1 µM ICI182780, 25 µM 7-KC+1 µM GW3965, 25 µM 7-KC+1 µM ECHS, 10nM E2, 1 µM ICI182780, 1 µM GW3965, 1 µM ECHS in complete growth media for 24 h at 37 °C. Antagonists were added 2 h before agonists' addition.

Animals and treatments

Male New Zealand white rabbits (1.5-2 years old, 3-5 kg), housed separately in cages in a room with 12 h dark/light cycle, were randomly assigned to 2 groups (n = 6 each) as follows: Group 1- Normal chow; Group 2- Chow

supplemented with 2 % cholesterol. Rabbits fed with cholesterol-enriched diet and their matched controls were euthanized with pentobarbital after 12 weeks. At necropsy, rabbits were perfused with Dulbecco's phosphate-buffered saline and the eyes were promptly enucleated. Retinal homogenates were made from one eye of each animal, and the other was immediately placed in a fixative solution for paraffin embedding. Retinal homogenates were used for Western blotting, ELISA, DCFH-DA, Amplex Red, and mass spectrometry studies. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Paraffin embedding of eyes

At necropsy, rabbits were perfused with Dulbecco's PBS and the eyes were promptly enucleated, and kept in alcoholic Z-fixative, then embedded in paraffin. 7 μm eye cross sections were made from paraffin embedded eyes. Sections were placed on slides, and used for immunohistochemistry.

Total cholesterol quantification

Total cholesterol levels in the control and cholesterol-fed rabbit retina samples were quantified by colorimetric detection using cholesterol/cholesteryl ester quantification kit as per the manufacturer's instructions. Cholesterol was extracted from the retina samples in a solution containing a mixture of chloroform: isopropanol: NP-40 (7:11:0.1). The extract was centrifuged at 15,000 $\times g$ for 10 min and the organic phase was transferred to a new tube. The organic phase liquid was air dried at 50 $^{\circ}\text{C}$ to remove chloroform and subjected to vacuum for 30 min to remove trace organic solvents. The dried lipids were

dissolved in 200 μL of cholesterol assay buffer provided with the kit until the samples were homogeneous by either sonicating or vortexing. Standards were prepared as per the manufacturer's instructions. 1 μL of the extracted sample adjusted to 50 μL / well with cholesterol assay buffer was used per assay. 50 μL of the reaction mix (containing 44 μL of cholesterol assay buffer, 2 μL of cholesterol probe, 2 μL of cholesterol enzyme mix and 2 μL of cholesterol esterase all provided in the kit) was added to each well containing standards and samples. After 1 h reaction at 37 °C the absorbance was read at 570 nm. The concentration of cholesterol in each sample was calculated using the standard curve and expressed as mg/g tissue.

Oxysterol levels measurement

Extracted retinas were shipped to Dr. David Russell's laboratory, the University of Texas Southwestern Medical Center at Dallas, TX, USA. Oxysterols were quantified in retinas from control (n = 3) and cholesterol-fed (n = 3) rabbits using a 4000 QTRAP liquid chromatography mass spectrometer (Applied Biosystems) as described in (McDonald, 2007).

Protein extraction

After treatment with indicated concentrations and times, cells were washed with Dulbecco's PBS, then harvested on ice with a mammalian protein extraction reagent (M-PER). Whereas tissue protein extraction reagent (T-PER) was used for extracting proteins from cholesterol-fed and control rabbit retinas. M-PER and T-PER were supplemented with protease and phosphatase inhibitors. Nuclear extracts were prepared by using NE-PER extraction kit. Bicinchoninic acid (BCA) assay was used for determining protein concentrations.

Equal amount of Laemmli sample buffer with 2-mercaptoethanol was added to the samples and were boiled for 10 min at 100 °C.

SDS-PAGE and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was used to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge). SDS binds to amino acids of the reduced protein and provides negative charge. Separation of the proteins by PAGE is mainly by size or molecular weight as they pass through acrylamide gel.

The Bio-Rad Mini-PROTEAN Tetra 4-gel vertical electrophoresis system was used for SDS-PAGE. A ready-to-pour SDS-PAGE acrylamide solution without a stacking gel was used. Various percentages of New Electrophoresis X'PRESS Technology (NEXT) gels were used for various molecular weight protein separations, described in the following table.

NEXT GEL 5 % Solution	30-500 kDa
NEXT GEL 7.5 % Solution	20-300 kDa
NEXT GEL 10 % Solution	10-200 kDa
NEXT GEL 12 % Solution	3.5-100 kDa

Ammonium persulfate (APS) and Tetramethylethylenediamine (TEMED) were added to catalyze the polymerization of acrylamide solutions into gel matrices. 10 µg samples and protein molecular weight marker were separated on SDS-PAGE gels at a constant voltage of 150V. 20X SDS-PAGE running buffer provided by the manufacturer was diluted to 1X in distilled water and used for electrophoresis separation. After protein separation, gels were equilibrated in

transfer buffer (25 mM Tris, 192 mM glycine) for 5 min. Polyvinylidene difluoride (PVDF) membranes are activated by methanol. Then gels were electrotransferred at constant voltage of 80 V for 2 h onto a PVDF membrane by using transfer buffer. After transfer, to block non-specific binding sites on the membrane surface, PVDF membranes were incubated in 5 % (5 g in 100 mL) non-fat milk solution in 1x T-TBS (25 mM Tris, 192 mM glycine, 0.5 % Tween-20) for 1 h at room temperature. After blocking, membranes were incubated overnight at 4 °C with primary antibodies at indicated concentrations.

Primary Antibodies used for Western blotting

Antibody	Dilution	Manufacturer
Mouse anti-ABCA-1	1:100	Neuromics, Edina, MN
Mouse Anti-BACE-1	1:1000	Millipore, Bedford, MD
Mouse anti-Bax	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse anti-Bcl-2	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse Anti-GADD153	1:1000	Abcam, Cambridge, MA
Mouse Anti-HO-1	1:500	Assay Designs, Ann Arbor, MI
Mouse Anti-NF- κ B	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse β -actin	1:5000	Santa Cruz Biotechnology, Santa Cruz, CA
Rabbit anti-CYP27A1	1:800	Protein Tech Group, Chicago, IL
Rabbit anti-IDE	1:5000	Millipore, Bedford, MD
Rabbit Anti-Lamin	1:500	Cell Signaling Technology, Inc. Danvers, MA

Rat Anti-caspase 12	1:1000	Abcam, Cambridge, MA
Rat anti-GFAP	1:200	Abcam, Cambridge, MA
Rabbit anti-ER α	1:500	Abcam, Cambridge, MA
Rabbit anti-ER β	1:200	Upstate, Lake Placid, NY, USA
Rabbit anti-LXR α	1:500	Abcam, Cambridge, MA
Mouse anti-LXR β	1:500	Abcam, Cambridge, MA

After overnight incubation at 4 °C with primary antibodies, membranes were washed for 6 times for 10 min with T-TBS. Membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h.

Secondary Antibodies used for Western blotting

Antibody	Dilution	Manufacturer
Goat anti-mouse HRP	1:5000	Bio-Rad, Hercules, CA
Goat anti-rabbit HRP	1:5000	Bio-Rad, Hercules, CA
Goat anti-rat HRP	1:2500	Santa Cruz Biotechnology, Santa Cruz, CA

After incubation, membranes were washed for 6 times for 10 min with T-TBS. Then the blots were developed with enhanced chemiluminescence Western blotting substrate immun-star HRP chemiluminescent kit. Bands were visualized on bioimaging system and analyzed by LabWorks 4.5 software (Upland, CA). Results were quantified by densitometry normalized to β -actin for cytosolic fractions or lamin A for nuclear fractions and analyzed as total integrated densitometric values (arbitrary units).

CytoTox-ONE Homogenous membrane integrity assay

The CytoTox-ONE homogenous membrane integrity assay is a homogenous, fluorometric method for assessing the number of non-viable cells. This test quantifies released lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH in the cell culture media was measured with a 10 min coupled enzymatic assay that results in the conversion of resazurin into resorfun. 30,000 ARPE-19 cells were seeded in 96 well plates. After confluency, cells were treated as described in ARPE-19 cell culture treatments for 24 h. After incubation, plates were allowed to equilibrate at room temperature for 20 min. 2 μ L Triton X-100 provided with the kit was added to positive control wells. CytoTox-ONE reagent was added to all samples and incubated for 10 min. The reaction was stopped with stop solution, included in the kit. The plate was read using a plate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The percentage of cytotoxicity was calculated by the following equation.

(Experimental-Culture medium background)

Percent cytotoxicity= 100 x-----

(Maximum LDH release-Culture medium background)

Enzyme-Linked Immunosorbent Assay

After treatments, conditioned media was collected. Protease and phosphatase inhibitors cocktail was added. The conditioned media was centrifuged at 16,000 \times g for 5 min at 4 $^{\circ}$ C. 100 μ L of supernatant was used for A β 1-42 and A β 1-40 quantification by colorimetric sandwich ELISA according to the manufacturer's instructions and as previously described (Prasanthi, 2009).

A β 1-42 and A β 1-40 levels were expressed in pg/mL. A β levels (A β 1-40 and A β 1-42) were quantified in the retina of control and cholesterol-fed rabbits with an ELISA kit according to the manufacturer's protocol. Briefly, to measure the amount of A β 1-40 and A β 1-42, the wet mass of the retina was homogenized thoroughly with 8 \times mass of cold 5 M guanidine HCl/50 mM Tris-HCl (pH=7.2). The homogenates were mixed for 3-4 h at room temperature and samples were diluted with cold reaction buffer (Dulbecco's phosphate-buffered saline with 5 % BSA and 0.03 % Tween-20 supplemented with 1 \times protease inhibitor cocktail) and centrifuged at 16,000 \times g for 20 min at 4 $^{\circ}$ C. The supernatant was decanted, stored on ice until use, diluted 1:2 with standard diluent buffer, and quantified by colorimetric sandwich ELISA kits. The quantity of A β in each sample was measured in duplicates. Protein concentrations of all samples were determined by standard BCA assay (Smith, 1985). A β levels were normalized to total protein content in the samples.

DCFH-DA assay for measuring reactive oxygen species

DCFH-DA, a nonfluorescent dye, is cleaved by esterase activity to yield DCFH, which is subsequently oxidized by a variety of ROS to form dichlorofluorescein (DCF), which is fluorescent. Because DCFH can be oxidized by various ROS, the increase of intracellular DCF fluorescence reflects an overall oxygen species index in cells. 30,000 ARPE-19 cells were seeded in 96 well plates. After confluency, cells were treated as described in ARPE-19 cell culture treatments for 24 h. After treatment, complete growth medium was removed and cells were washed with PBS. 5 μ M DCFH-DA dissolved in the complete growth medium was added to cells.

Fluorescence was read at excitation and emission wavelengths of 488 nm and 525 nm, respectively at 0 min, 15 min, 30 min, and 60 min.

ROS generation was measured in retinal tissue homogenates using DCFH-DA as previously described (Bejma, 2000; Bejma and Ji, 1999; Rush, 2007). Retinas were homogenized in T-PER using a glass homogenizer. Samples containing 25 µg proteins diluted in Dulbecco's PBS were incubated with 5 µM DCFH-DA. Fluorescence was measured at 0 min and every 15 min for 1 h at excitation and emission wavelengths of 488 nm and 525 nm, respectively, using a microplate reader. Values were expressed as relative fluorescence units (RFU) normalized per mg of protein.

Amplex Red Hydrogen Peroxide/Peroxidase Assay

The Amplex Red hydrogen peroxide/oxidase assay uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H₂O₂ or peroxidase activity (Zhou, 1997). In the presence of peroxidase, the Amplex Red reagent reacts with H₂O₂ in a 1:1 stoichiometry and produces the red-fluorescent oxidation product, resorufin.

Retinal homogenates of control and cholesterol-fed rabbits are diluted in reaction buffer and added into 96 well plates. For each well 50 µL of working solution containing 100 µM Amplex Red reagent and 0.2 U/mL HRP was added and after 30 min incubation in dark, fluorescence was measured. H₂O₂ standard curve was generated from 0 µM to 5 µM and H₂O₂ concentrations of retina samples were calculated from the standard curve.

Similarly, for peroxidase activity determination, 100 µM Amplex Red reagent containing 2 mM H₂O₂ was added to retinal homogenates and after

incubation fluorescence was measured. Peroxidase standard curve was generated in the range from 0 to 2 mU/mL. Peroxidase activity of retinal samples was calculated from standard curve. Resorufin fluorescence was measured using a microplate reader with excitation at 530-560 nm and emission at 590 nm.

JC-1 Mitochondrial Membrane Potential Detection

Apoptosis involves genetically programmed series of events leading to cell death. During this process several key events occur in mitochondria including the loss of mitochondrial membrane potential (Green and Reed, 1998) which is an early event preceding phosphatidylserine externalization and coinciding with caspase activation. Studies have shown that apoptotic cells are present in the RPE of eyes with AMD, indicating that apoptosis of RPE cells could contribute to AMD (Cai, 1999; Ishibashi, 1986b). Fluorescent cationic dye, JC-1 was used to measure the loss of mitochondrial membrane potential (Smiley, 1991).

In non-apoptotic cells, JC-1 accumulates as aggregates in the mitochondrial membranes, resulting in red fluorescence (590 nm) (Cossarizza, 1993). In apoptotic cells, JC-1 exists in the green fluorescent (529 nm) monomeric form because of decreased mitochondrial membrane potential. JC-1 is more specific for mitochondrial versus plasma membrane potential, and more consistent in its response to depolarization, than other cationic dyes such as DiOC6 (3) and rhodamine 123 (Salvioli, 1997).

30,000 ARPE-19 cells were plated in 96 well plates. After confluency, cells were treated as described in ARPE-19 cell culture treatments for 24 h. After incubation, media was aspirated and cells were washed with Dulbecco's PBS. 100 μ L of 1X JC-1 reagent was added to cells, and incubated at 37 °C for 30 min. After

incubation, cells were washed with PBS. 100 μ L PBS was added to cells. Red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) was measured by a microplate reader. The ratio of red fluorescence divided by green fluorescence was determined. The ratio of red to green is decreased in dead cells and in cells undergoing apoptosis compared to healthy cells.

The DeadEnd Fluorometric TUNEL System for apoptotic cell Detection

The DeadEnd fluorometric TUNEL system was used for detection of apoptotic cells. Nuclear DNA fragmentation is an important biochemical hallmark of apoptosis. Endogenous nucleases breakdown DNA into fragments (Oberhammer, 1993; Schwartzman and Cidlowski, 1993). The TUNEL system measures fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail using the principle of the TUNEL assay.

ARPE-19 cells were grown and treatments were carried out. After treatments, cells were fixed with 4 % paraformaldehyde in Dulbecco's PBS at room temperature. Whereas retinal sections were deparaffinized, rehydrated and washed with PBS. Slides were permeabilized with 0.1 % Triton X-100, and then washed with PBS. Equilibration buffer was added and covered with plastic coverslip. Incubation buffer containing equilibration buffer, nucleotide mix and rTdT enzyme were added and incubated at 37 °C for 1 h. After incubation, slides were dipped in saline-sodium citrate (SSC) buffer. Slides were washed with

Dulbecco's PBS, and stained with Propidium iodide. The fluorescein-12-dUTP-labeled DNA was visualized by confocal microscope coupled to a Zeiss Axiophot 200 inverted epifluorescence microscope.

GSH-Glo Glutathione Assay

GSH is a tripeptide (L- γ -glutamyl-L-cysteinyl-glycine) antioxidant found in eukaryotic cells (Griffith, 1999; Pompella, 2003; Sies, 1999). A drop in GSH levels either by oxidation or reaction with thiol group can be caused by reactive chemical species. A change in GSH levels potentially leads to apoptosis and cell death (Townsend, 2003). For detection and quantification of GSH, the GSH-Glo glutathione assay was used. It is a luminescence-based assay. In the presence of GSH, a luciferin derivative is converted into luciferin by glutathione S-transferase (GST). The signal produced in a coupled reaction with firefly luciferase is relative to the amount of glutathione present in the sample.

30,000 ARPE-19 cells were plated in 96 well plates. After confluency, cells were treated as described in ARPE-19 cell culture treatments for 24 h. After incubation the media was removed. GSH-Glo reagent was added and the plate was incubated for 30 min at room temperature. Reconstituted luciferin detection reagent was added to the plate, which was again incubated for another 15 min at room temperature. A microplate reader was used to read luminescence, which is proportional to the amount of GSH present in the sample. GSH standard curve was generated by diluting 5 mM stock. 0 μ M to 5 μ M range standards were used to quantify the amount of GSH present in the sample.

Calcium Imaging

ARPE-19 cells were cultured on 35 mm glass bottom culture dishes and incubated with 0, 10 or 25 μM of 27-OHC for 24 h. To measure intracellular Ca^{2+} concentration, cells were loaded with 2 μM fura-2AM for 45 min at 37 °C under an atmosphere of 5 % CO_2 -95 % air, washed three times with Ca^{2+} -free SES buffer (Panini and Sinensky, 2001). For fluorescence measurements, the fluorescence intensity of Fura-2AM -loaded cells were monitored using a charge-coupled device (CCD) camera-based imaging system mounted on an Olympus XL70 inverted microscope equipped with an Olympus 40 \times (1.3 NA) fluor objective. A monochromator dual wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm with an Orka imaging camera. The images of multiple cells collected at each excitation wavelength were processed using the Ca^{2+} imaging, PCI software to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that of excitation at 380 nm (F_{340}/F_{380}).

Immunocytochemistry, Immunohistochemistry and H&E Staining

ARPE-19 cells were grown on collagen coated coverslips. After confluency, cells were treated as described in ARPE-19 cell culture treatments for 24 h. After incubation cells were washed with PBS, fixed in 4 % paraformaldehyde, blocked with 5 % normal goat serum and incubated overnight at 4 °C with monoclonal mouse antibodies to GADD153 or NF- κB p65. Cells were washed with PBS, incubated with secondary antibodies conjugated to Alexa fluor-488 for 1 h at room temperature, washed with PBS, mounted with mounting medium and

visualized with a Zeiss LSM 510 META confocal system coupled to a Zeiss Axiophot 200 inverted epifluorescence microscope.

7 μm paraffin sections of eyes were deparaffinized with xylene and rehydrated with series of percentages of ethanol. Slides were kept in a rack and performed following washes.

1: 4 times, each 2 min with Xylene

2: 2 times, each 3 min with 100 % Ethanol

3: 3 min with 95 % Ethanol

4: 3 min with 70 % Ethanol

5: 3 min with 50 % Ethanol

6: Running cold tap water to rinse

After that slides were washed with PBS, incubated with trypsin enzymatic antigen retrieval solution, then blocked with 10 % normal goat serum solution for 1 h. This was followed by an overnight incubation at 4 °C with primary antibodies. Slides were washed with PBS 3 times for 5 min each. Secondary antibodies were incubated in PBS for 1 h at room temperature. Slides were washed with PBS 3 times for 5 min each. DAPI or propidium iodide was used as a counter stain for visualizing nuclei. Routine H&E staining was carried out after deparaffinization and rehydration of retinal sections with xylene, ethanol and deionized H₂O. Antibodies and dyes used for experiments are listed below.

Antibodies/Dyes

Antibody / Dye	Dilution	Manufacturer
Mouse anti-GADD153	1:250	Abcam, Cambridge, MA

Mouse NF- κ B p65	1:250	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse anti- β amyloid, 1-16(6E10)	1:100	Signet laboratories Inc., Dedham, MA
Alexa Fluor 488 goat anti-mouse IgG	1:500	Molecular Probes, Inc., Eugene, OR
Rat anti-GFAP	1:50	Abcam, Cambridge, MA
Mouse anti-CYP27A1	1:200	Novus Biologicals, Littleton, CO
Alexa Fluor 594 goat anti-rat IgG	1:500	Molecular Probes
Trypsin enzymatic antigen retrieval solution	1:1	Abcam, Cambridge, MA
Vectashield mounting medium – DAPI	NA	Vector laboratories, Inc., Burlingame, CA
Vectashield mounting Medium Propidium Iodide	NA	Vector laboratories, Inc., Burlingame, CA

Transfection and Dual-Luciferase Reporter Assays

ARPE-19 cells were plated in 96 well plates. Estrogen transcriptional response element (ERE) reporter (a mixture of ERE-responsive firefly luciferase construct and renilla luciferase construct, 40:1), a negative control (a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1), and a positive control (a mixture of constitutively expressing green fluorescent protein (GFP), constitutively expressing firefly luciferase, and constitutively expressing renilla luciferase constructs, 40:1:1) were diluted in Opti-MEM serum free media.

For testing LXR activity, liver X receptor response element (LXRE) reporter (a mixture of inducible LXR-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1), a negative control (a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1), a positive control (a mixture of constitutively expressing GFP, constitutively expressing firefly luciferase, and constitutively expressing renilla luciferase constructs, 40:1:1) were diluted in OptiMEM serum free media.

Lipofectamine LTX and PLUS reagents were used for transfection, and was carried out according to the manufacturer's protocol. After 24 h, media was replaced with DMEM/F12 media, and transfected ARPE-19 cells were treated with E2, 27-OHC, 25-OHC and 7-KC for ER activity and GW3965, 27-OHC, 25-OHC and 7-KC for LXR activity determination. After 24 h treatment at 37 °C, cells were harvested into cell lysis buffer. Firefly and renilla luciferase activities were determined by using Dual-Luciferase assay system and microplate reader. Promoter activity values are expressed as arbitrary units using a renilla reporter for internal normalization.

Statistical Analysis

GraphPad Prism software 4.01 was used for statistical analysis. Quantitative data are presented as mean values \pm SEM. The significance of differences between the control and cholesterol-treated group was assessed by unpaired Student's *t* test, with $P < 0.05$ considered statistically significant. The significance of differences among the treatments was assessed by one-way analysis of variance (one-way ANOVA) followed by Tukey's *post hoc* test. All values obtained from the three

different experiments were expressed as mean value \pm SEM. $p < 0.05$ was considered statistically significant.

CHAPTER III

RESULTS

A β levels were increased in retinas of cholesterol-fed rabbits

A β is found in drusen, the extracellular deposits that are the earliest sign of AMD. Drusen accumulates between the basal surface of RPE and Bruch's membrane. A β deposition could be an important element in local inflammatory outcome that contribute to deterioration of photoreceptors and pathogenesis of AMD. Drusen deposition is accompanied with degeneration of photoreceptor cells. Immunohistochemical analysis in the present study with laser scanning confocal microscopy showed increased immunoreactivity to A β peptide in retinas from the cholesterol-fed rabbits compared to control rabbits as determined with 4G8 antibody (Figure 12A). A β monoclonal antibody 4G8 is reactive to amino acid residues 17-24 of A β and also reacts with APP. The increase in A β staining is observed in the photoreceptor outer segments, outer nuclear layer, inner nuclear layer and also in the ganglion cell layers.

A β quantitation by ELISA (Figure 12B, C) also showed a significant increase in both A β ₁₋₄₀ (Figure 12B) and A β ₁₋₄₂ (Figure 12C) forms in the retinal samples of cholesterol-fed rabbits when compared to controls. A β levels are regulated by generation from APP upon initial cleavage by BACE-1 and degradation by IDE. Western blot analyses (n=3 control; n=3 cholesterol-fed)

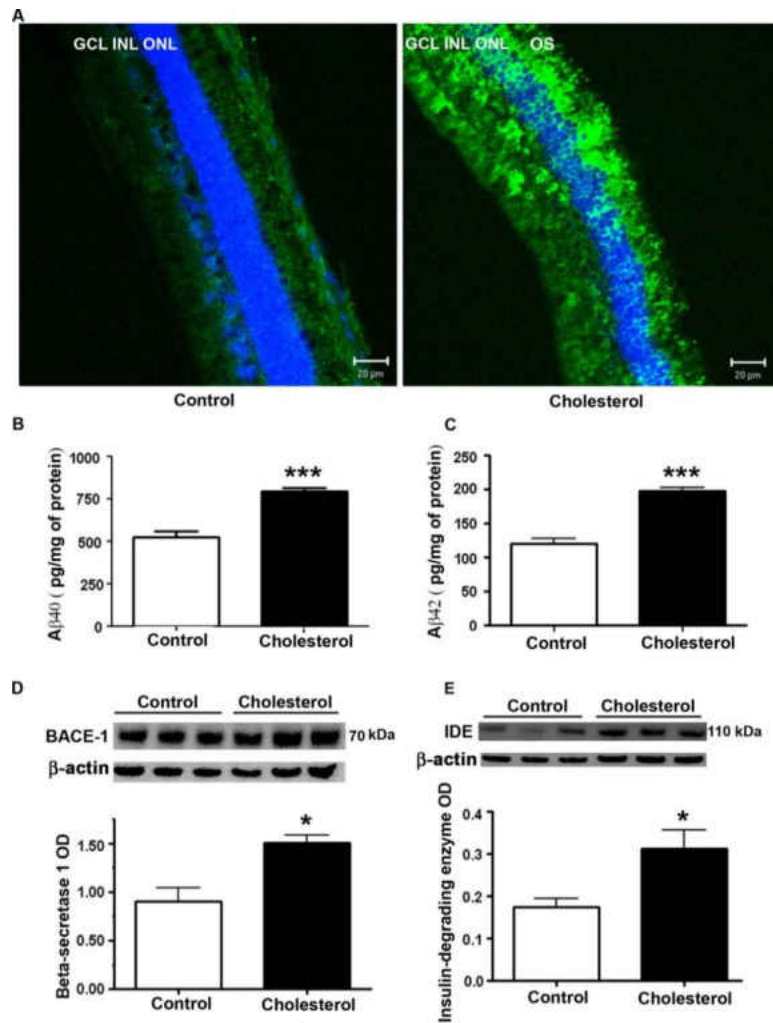


Figure 12: 4G8 immunoreactivity and A β levels were increased in cholesterol-fed rabbit retina

A. Immunohistochemical analysis of retinal sections with 4G8 (green), an antibody that detects A β , showed increased immunoreactivity in the cholesterol-fed rabbits compared to control. DAPI (blue) is a nuclear counter stain. Bar = 20 μ m. OS, ONL, INL, GCL are outer segments, outer nuclear layer, inner nuclear layer and ganglion cell layers respectively. B, C. A β quantification by ELISA showed an increase in levels of A β 1-40 and 1-42 in the retinal samples of cholesterol-fed rabbits compared to normal rabbits. D. BACE-1 and E. IDE levels are increased in retinas from cholesterol-fed rabbits, compared to controls. *p < 0.05, ***p < 0.001 vs control.

demonstrate that BACE-1 and IDE levels were significantly increased in cholesterol-fed rabbit retinas, compared to their respective controls (Figure 12D, E).

These results suggest that both formation and degradation of A β are enhanced by the cholesterol enriched diet and that the increase in generation by beta-secretase 1 exceeds the degradation rate of A β peptide by Insulin-degrading enzyme.

Cholesterol-fed rabbit retinas show increased oxidative stress

As A β is a neurotoxic peptide and its accumulation in the retina may promote oxidative damage and cell death, the extent to which accumulation of A β peptide is associated with increased ROS and apoptotic cell death was determined. In 1954 Science paper, Gershman's free radical theory of oxygen toxicity for the first time showed harmful side of oxygen (Gerschman, 2001). Oxidative stress has been implicated in many degenerating diseases including AD and AMD (Crabb, 2002; Hawkins, 1999; Markesbery, 1997; Shen, 2007; Zarbin, 2004).

The cholesterol-enriched diet significantly increased ROS levels in retinas (n=6) compared to control rabbits (Figure 13A). Fluorometric detection of H₂O₂ and peroxidase by amplex red assay showed significant increases in H₂O₂ levels and decreases in peroxidase activity in retinas from cholesterol-fed rabbits compared to retinas from control rabbits (Figure 13B, C). Levels of the oxidative stress sensor HO-1 were determined by Western blotting. The levels of HO-1 were significantly higher in retinas (n=3 each) of cholesterol-fed compared to normal

chow-fed rabbits (Figure 13D). All together, these results show that retinas from cholesterol-fed rabbits were subjected to oxidative stress.

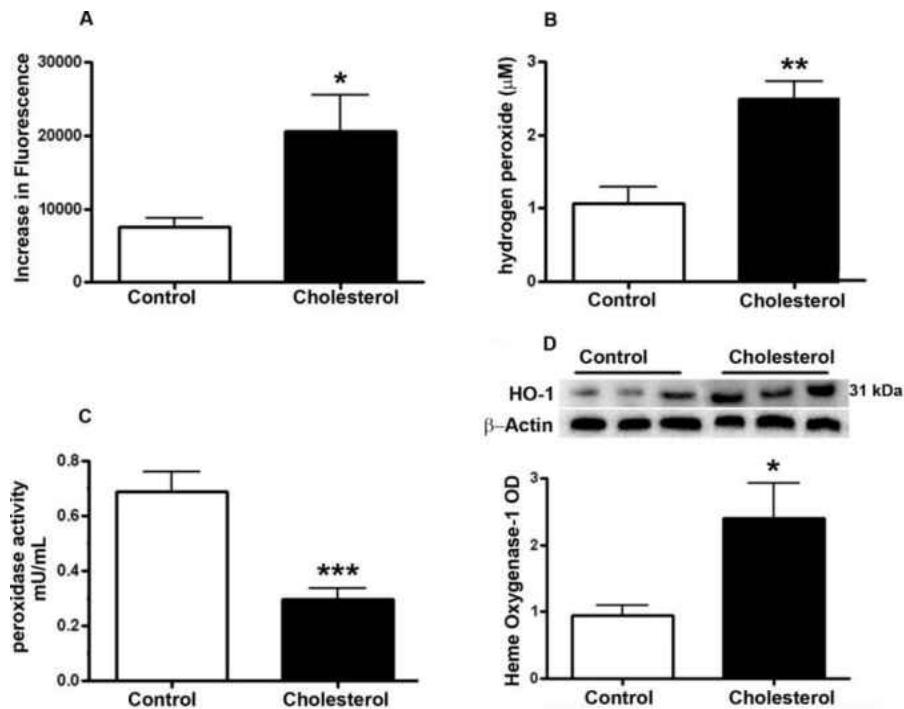


Figure 13: Retinas of cholesterol-fed animal exhibit an increase in oxidative stress.

A. The cholesterol-enriched diet significantly increased ROS levels in the retinas as demonstrated by DCFH-DA. B, C. Fluorometric detection of H₂O₂ and peroxidase activity by Amplex Red assay showed an increase in H₂O₂ levels in cholesterol-fed rabbit retinas and decreased peroxidase activity. D. HO-1 levels were increased in the retinas of cholesterol-fed rabbits as shown by Western blotting. *p < 0.05; **p < 0.01; ***p < 0.001 vs control.

Cholesterol-enriched diet caused retinal morphological changes

Astrogliosis is one of the remarkable characteristics of astrocytes to respond to oxidative stress insults. Confocal microscopy analysis show significant astrogliosis, as detected by increased immunoreactivity to glial fibrillary acidic

protein (GFAP) in retinas from cholesterol-fed rabbits compared to control rabbits (Figure 14A). The increase in the number of astrocytes occurred in the outer nuclear, and the ganglion cell layers. Western blot analyses also show that levels of GFAP are dramatically increased in retinas from cholesterol-fed rabbits compared to control rabbits (Figure 14B).

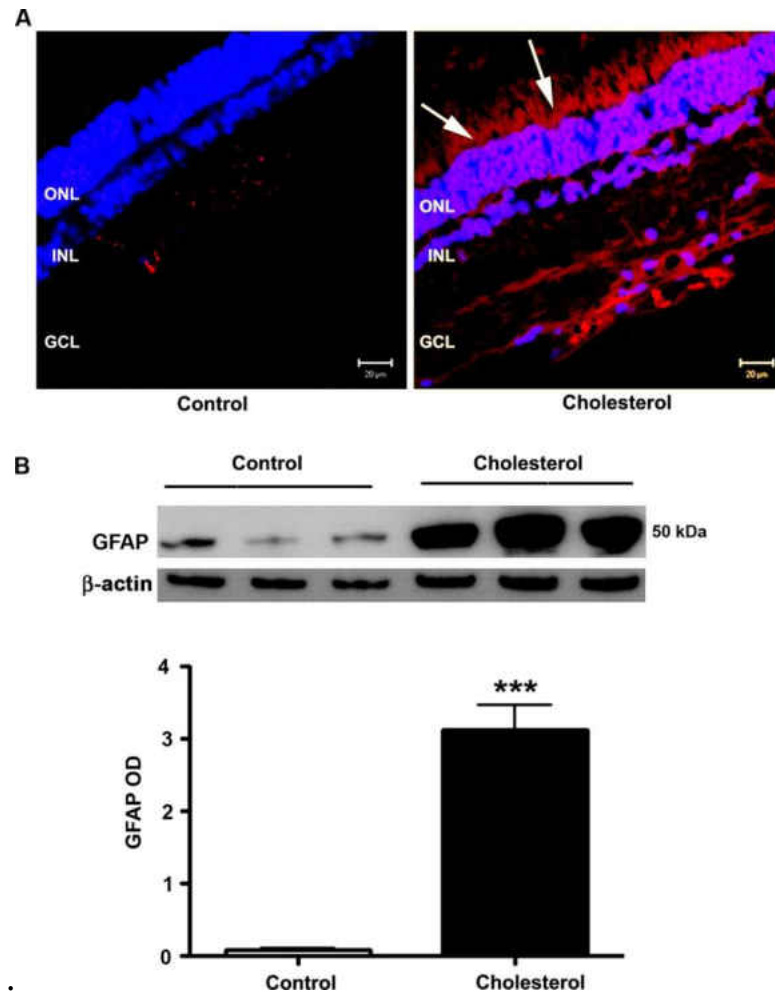


Figure 14: Cholesterol-enriched diet increased GFAP expression in retina.

A. Cholesterol-fed rabbit retinal sections showed an increase in the expression of GFAP (Red). DAPI (blue) is a nuclear counter stain. B. Western blot (n=3 each) results also showed elevated GFAP levels. ONL=outer nuclear layer, INL= inner nuclear layer GCL= ganglion cell layers. ***p < 0.001 vs control. Bar = 20 μ m.

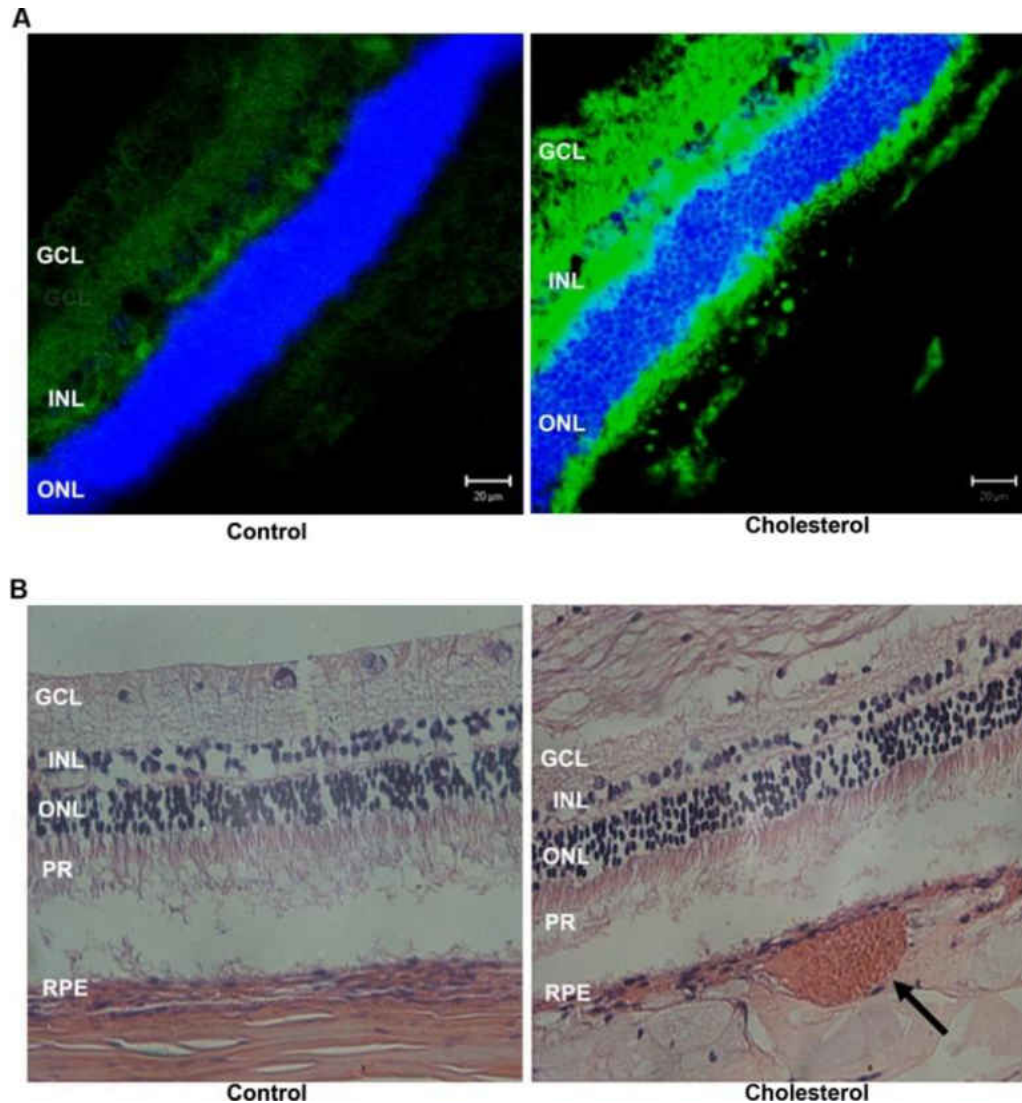


Figure 15: Cholesterol-enriched diet caused retinal morphological changes

A. Confocal microscopy showed an increase in immunoreactivity of vitronectin antibody (green) in the retinas of rabbits fed with cholesterol-enriched diet compared to rabbits fed with normal chow. B. Drusen like debris was detected under the retinal pigment epithelium (arrow) of cholesterol-fed rabbits. GCL=Ganglion cell layer; INL=Inner nuclear layer; ONL=Outer nuclear layer; PR=Photoreceptors; RPE=Retinal pigment epithelium. Bar = 20 μ m

Vitronectin, an adhesive glycoprotein, is a common component of extracellular matrices in adult tissues including Bruch's membrane

(Hageman, 1999) and is expressed in retina. Confocal microscopy showed an increase in immunoreactivity for vitronectin antibody in the retinas of rabbits fed with cholesterol-enriched diet compared to rabbits fed with normal chow (Figure 15A). H&E staining analyzed by light microscopy, showed necrotic debris suggestive of drusen-like debris in retinas from cholesterol-fed rabbits (Figure 15B). These drusen-like debris are localized under the RPE.

Cholesterol-enriched diet caused apoptotic cell death

Western blot results show that levels of the anti-apoptotic protein Apoptosis regulator Bcl-2 were decreased and levels of the pro-apoptotic protein apoptosis regulator BAX were increased in retinas of cholesterol-fed rabbits in comparison to levels of these proteins in retinas of control rabbits. Bcl-2 to Bax ratio of cholesterol fed rabbits is significantly reduced when compared to control rabbits (Figure 16A). In addition to Bcl-2 and Bax, levels of GADD153 were significantly increased in cholesterol-fed rabbits (Figure 16B). Growth arrest and DNA damage-inducible protein GADD153 (also called CHOP), a transcription factor that is activated by stress to the endoplasmic reticulum, triggers cell death by mechanisms that may include generation of ROS, downregulation of Bcl-2, and upregulation of Bax (Oyadomari and Mori, 2004).

These results indicate that stress to the endoplasmic reticulum is involved in the deleterious effects of cholesterol-enriched diet in retinas of rabbits. TUNEL assay detects the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the terminal deoxynucleotidyl transferase.

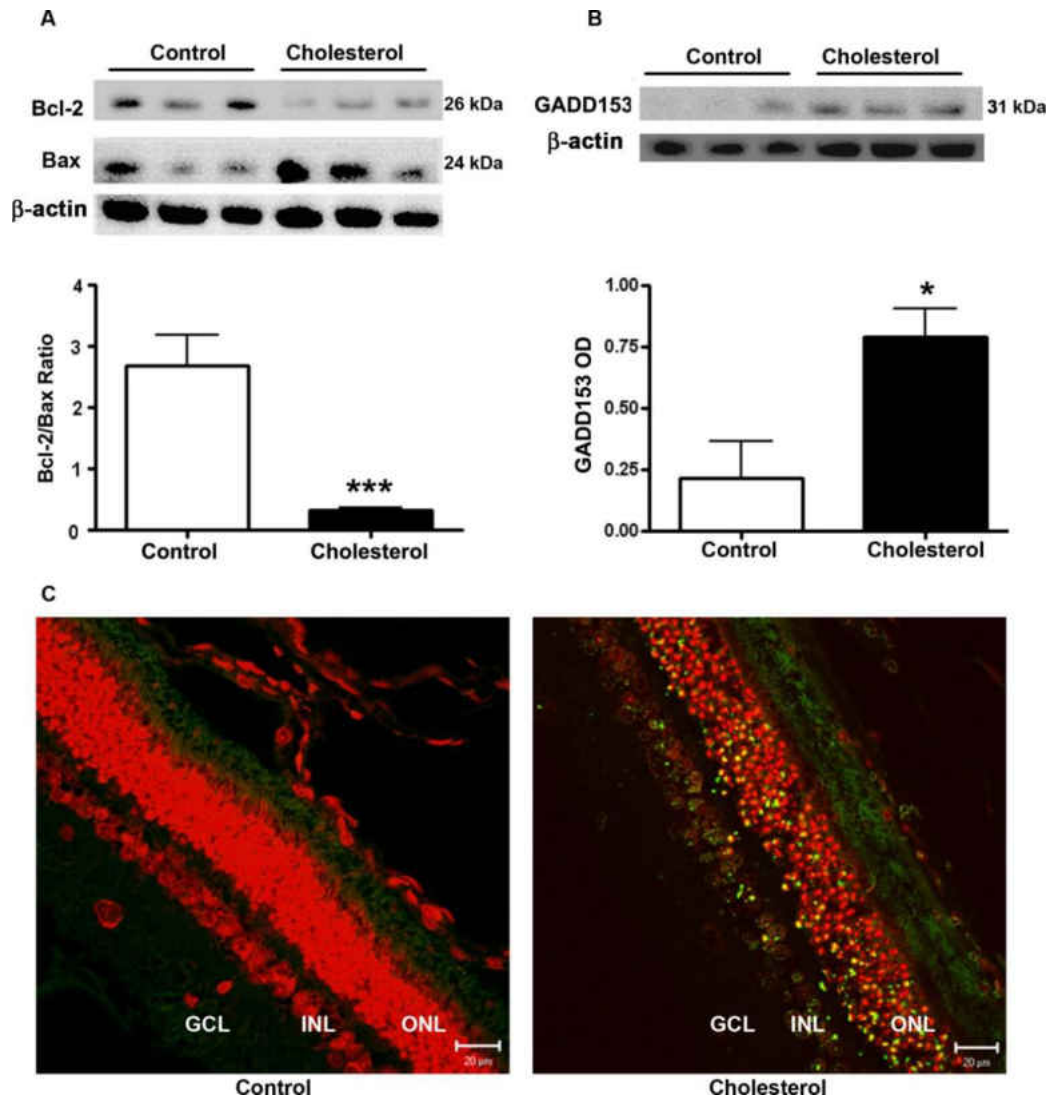


Figure 16: Cholesterol-enriched diet caused apoptotic cell death in rabbit retina. **A.** Western blot results showed a decrease in the levels of the anti-apoptotic protein Bcl-2 and an increase in levels of the pro-apoptotic protein Bax levels in the retinas of rabbits fed with cholesterol-enriched diet compared to control rabbits. **B.** Levels of the endoplasmic reticulum stress marker GADD153 were increased in cholesterol-fed rabbits as shown by Western blotting. **C.** While no TUNEL-positive cells were detected in retinas from control rabbits, a large number of TUNEL-positive cells were observed in retinas of cholesterol-fed rabbits. Propidium iodide (red) was used as a counterstain for nuclei. Bar = 20 μ m. * $p < 0.05$, *** $p < 0.001$ vs control.

TUNEL staining showed no apoptotic cells in the control retinas, whereas an extensive staining was observed in the retinas of cholesterol-fed rabbits (Figure 16C). Propidium iodide was used as nuclear counter stain. Yellow color is the merge of green color which indicates fragmented DNA and red color which indicates nucleus.

Cholesterol-enriched diet disturbed cholesterol homeostasis in the retina

The extent to which cholesterol-enriched diet increases the accumulation of cholesterol and major oxysterols were determined in rabbit retinas (Table 1). Total cholesterol levels were increased in cholesterol-fed rabbit retinas compared to control retina. The amount of cholesterol in control retinas was 0.1024 ± 0.028 mg/g of tissue, whereas in cholesterol-fed rabbit retina the concentration of cholesterol increased to 0.8427 ± 0.002 mg/g of tissue. Cholesterol is also eliminated through enzymatic conversion to polar oxysterols that include 27-OHC, 24-OHC, and 22-hydroxycholesterol (22-OHC) by sterol 26-hydroxylase, mitochondrial (CYP27A1), Cytochrome P450, family 46, subfamily A, polypeptide 1 (CYP46A1), and cholesterol side-chain cleavage enzyme, mitochondrial (CYP11A1) respectively. All these enzymes were found to be expressed in the retina (Rodriguez and Larrayoz, 2010). Mass spectrometry analysis showed a significant increase in the levels of oxysterols 27-OHC, 24-OHC, and 22-OHC in cholesterol-fed rabbit retinas (Table-1).

Other enzymatically-generated oxysterols, 7α -hydroxycholesterol (7α -OHC), 4β -hydroxycholesterol (4β -OHC) and 25-OHC were also measured and found to be increased in their levels in retinas from cholesterol-fed rabbits

(Table-1). CYP27A1, the enzyme that converts cholesterol to 27-OHC, levels were investigated by Western blotting and immunohistochemistry (Figure 17).

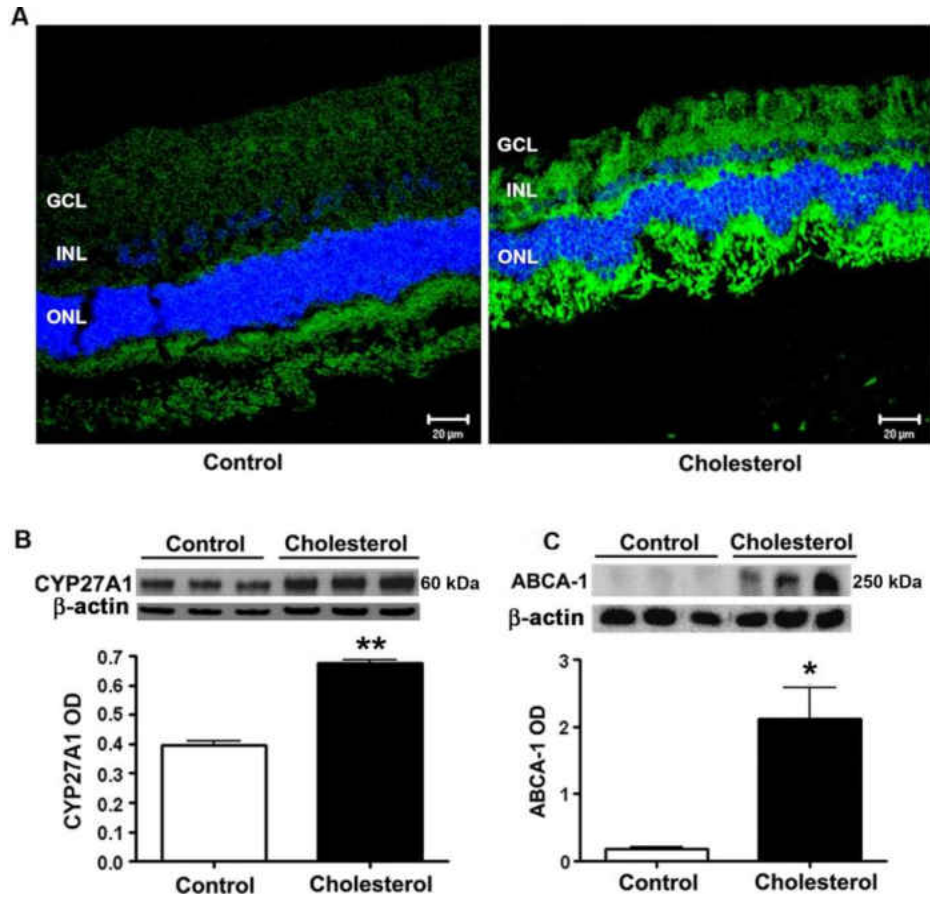


Figure 17: Cholesterol-enriched diet disturbed cholesterol homeostasis in the retina.

A. Cholesterol-fed rabbit retinal sections showed an increase in the expression of CYP27A1 (green). DAPI (blue) is a nuclear counter stain. ONL, INL, GCL are outer nuclear layer, inner nuclear layer and ganglion cell layers respectively. B. Western blotting further confirmed an increase in CYP27A1 expression. C. Western blotting results showed that ABCA-1 levels were increased in the retinas of cholesterol-fed rabbits. * $p < 0.05$, ** $p < 0.01$ vs control. Bar 20 μm .

CYP27A1 immunoreactivity was dramatically increased in retinas from

cholesterol-fed rabbits compared to control rabbits (Figure 17A).

Table 1 - Total cholesterol and oxysterol levels in control (n=3) and cholesterol-fed rabbit retina (n=3)

	Control retina	Cholesterol-fed retina
Total cholesterol mg/g tissue	0.1024 ± 0.028	0.8427 ± 0.002 ***
4β-hydroxycholesterol ng/g tissue	2491 ± 236	9046 ± 1015 **
7α-hydroxycholesterol ng/g tissue	344.1 ± 49.51	14378 ± 1504 **
22-hydroxycholesterol ng/g tissue	7.893 ± 1.59	105.6 ± 24.71 *
24-hydroxycholesterol ng/g tissue	405.3 ± 173.9	1815.32 ± 313.5 *
25-hydroxycholesterol ng/g tissue	24.39 ± 0.20	835.5 ± 29.77 ***
27-hydroxycholesterol ng/g tissue	29.38 ± 8.4	697.2 ± 161.4 *

Values are expressed as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001 vs control.

Western blotting also showed a significant increase in CYP27A1 levels in retinas from cholesterol-fed rabbits compared to those in retinas of control rabbits (n=3 each; Figure 17B). The cholesterol transporter ATP-binding cassette sub-family A member 1 (ABCA-1) shuttles cholesterol between various cells. Levels of ABCA-1 were dramatically increased in retinas of cholesterol-fed rabbits in comparison to levels in retinas of control rabbits (n=3 each; Figure 17C). All together, these results demonstrate accumulation of cholesterol and increased conversion to oxysterols in retinas as a result of cholesterol-enriched diet.

Previous research from our laboratory has shown that 27-OHC can cause AD like pathology in organotypic slices of rabbit brain and neuroblastoma cell line SHSY-5Y (Prasanthi, 2009; Sharma, 2008). Whether the oxysterol 27-OHC can cause pathological hallmark features related to AMD in retinal pigment epithelial cell line was investigated here. 27-OHC can increase A β levels in organotypic slices of rabbit brain and SHSY-5Y cells. If 27-OHC has similar effect on A β , a major component of drusen, in retinal pigment epithelial cells was investigated by using ARPE-19 cells.

27-OHC increased A β production

RPE cells secrete a variety of extracellular matrix proteins, complement factors, and protease inhibitors that have been reported to be major constituents of drusen (Farkas, 1971). APP immunoreactivity present in the RPE cytoplasm, anti-A β labeled structures identified in RPE cells and cultured human RPE cells labeled with APP and A β antibodies strongly suggest an RPE origin of amyloid. RPE has the ability to synthesize significant quantities of APP and generate A β . Amyloid vesicles in drusen are derived from degenerate RPE cells that contain A β and other molecules.

Beta-secretase 1 (BACE-1) is responsible for proteolytic processing of APP. BACE-1 is necessary for A β production *in vivo* and is the rate limiting step in the production of A β (Luo, 2001). A β is a primary component of amyloid plaques. Amyloid plaques and neurofibrillary tangles are two defining pathological hallmarks of AD. AMD hallmark component drusen comprises of A β (Anderson, 2004; Dentchev, 2003; Johnson, 2002). After 27-OHC treatment, spent media of ARPE-19 cells was used for A β levels quantification.

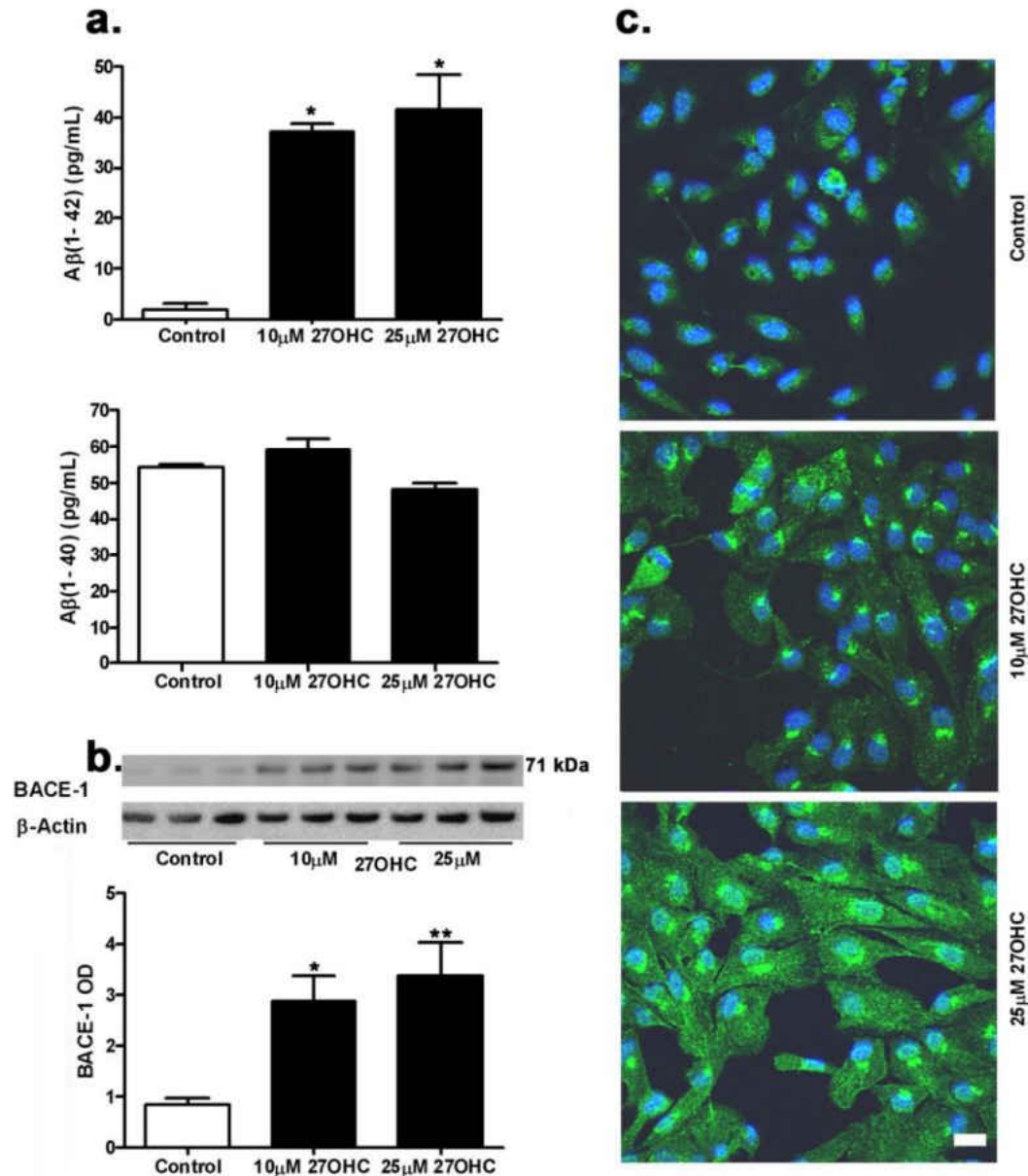


Figure 18: 27-OHC increased A β production in ARPE-19 cells

A. Treatment of ARPE-19 cells with 10 μ M and 25 μ M 27-OHC for 24 h increased A β 1-42 but not A β 1-40 levels. B. Levels of BACE-1, the enzyme that initiates the generation of A β , are also increased following treatment with 27-OHC. C. Immunocytochemistry with 6E10, an antibody that detects A β , shows increased staining with 27-OHC (green); DAPI (blue) was used as a nuclear counterstain (c). * $p < 0.05$, ** $p < 0.01$ vs control; Bar, 20 μ m.

Treatment with 27-OHC at 10 μM and 25 μM for 24 h significantly increased A β ₁₋₄₂ but not A β ₁₋₄₀ levels in these cells. The magnitude of increase in A β ₁₋₄₂ levels is similar with 10 μM and 25 μM of 27-OHC (Figure 18A). Both 10 μM and 25 μM 27-OHC significantly increased BACE-1 expression levels (Figure 18B), suggesting that the increased levels of A β ₁₋₄₂ derive, at least in part, from an increase in the rate of production of this peptide through the processing of APP by BACE-1. The immunocytochemistry using 6E10 antibody that detects A β peptides also showed an increase in the A β immunoreactivity with both 10 μM and 25 μM of 27-OHC compared to untreated cells (Figure 18C). The A β staining appeared to be mostly perinuclear. Increased A β ₁₋₄₂ peptide levels in the ARPE-19 cells could cause oxidative damage to these cells, as high levels of this peptide either soluble or insoluble form is toxic to cells.

27-OHC disturbed Ca²⁺ homeostasis and affected endoplasmic reticulum stress

Ca²⁺ is essential for cell functioning, and cell survival depends on the maintenance of Ca²⁺ homeostasis (Selvaraj, 2009). Disruptions of Ca²⁺ homeostasis result in the development of the endoplasmic reticulum stress response which can compromise cell survival.

The addition of thapsigargin (Tg, 2 μM), which initiates release of Ca²⁺ from the internal endoplasmic reticulum stores (in Ca²⁺ free media) caused a significant decrease in endoplasmic reticulum Ca²⁺ content in cells treated with either 10 or 25 μM of 27-OHC. Importantly, addition of 1 mM Ca²⁺ externally showed a robust increase in cytosolic Ca²⁺ levels in control cells.

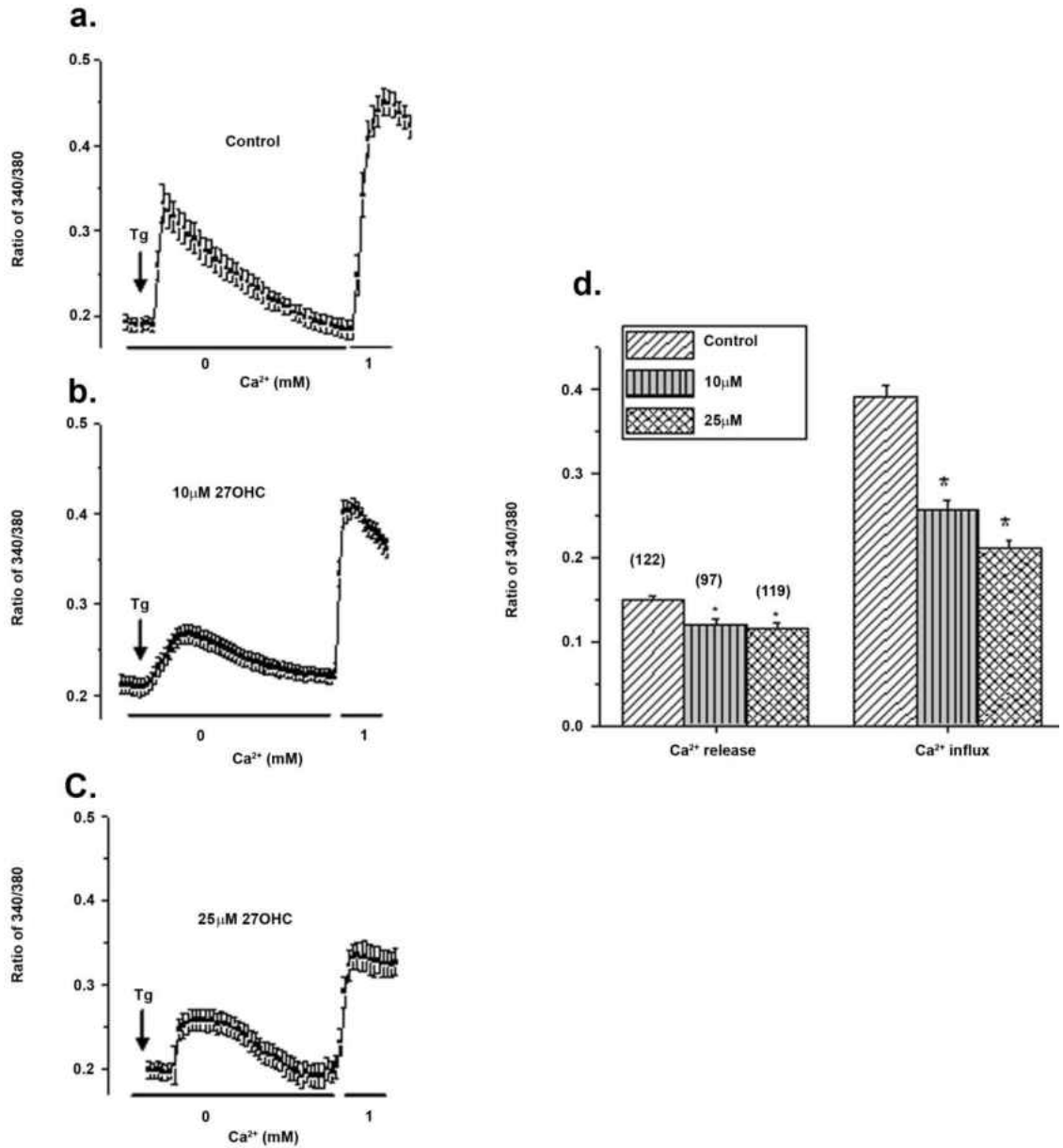


Figure 19: Ca²⁺ homeostasis is disturbed by 27-OHC in ARPE-19 cells

A. Ca²⁺ imaging was performed in the presence of thapsigargin (Tg; 2 μ M in a Ca²⁺-free media) in control ARPE-19 cells or B. cells treated with 10 μ M or C. 25 μ M 27-OHC. D. Ca²⁺ influx was measured by the addition of 1 mM Ca²⁺ externally and traces shown here are averages of 30-40 cells in each condition. Bar graph indicates the mean values of the first peak (endoplasmic reticulum calcium release) and second peak (calcium entry). *p < 0.05 vs control.

However, cells treated with 10 or 25 μM 27-OHC showed a gradual decrease in Ca^{2+} entry (40-50 % decrease) (Figure 19A, B, C). Mean Ca^{2+} influxes from 90-120 individual cells are shown as bar graph (Figure 19D). Basal Ca^{2+} influx (measured upon addition of external Ca^{2+} without Tg stimulation) was not altered. Overall, these data demonstrate that treatment of the cells with 27-OHC leads to a decrease in intracellular Ca^{2+} entry and thus decreased endoplasmic reticulum Ca^{2+} . This could lead to endoplasmic reticulum stress, since Ca^{2+} entry through the plasma membrane is essential for the refilling of the endoplasmic reticulum store. As a result of endoplasmic reticulum stress, caspase-12 is activated and can lead to apoptosis (Cordeiro, 2010). Western blot results showed that 27-OHC treatment increased the levels of caspase-12 (Figure 20A).

Caspase-12 is a initiator caspase and is activated by endoplasmic reticulum stress (Nakagawa, 2000). Once activated, it induces the cleavage of caspase-3 in a cytochrome c-independent manner. Activation of caspase-12 during apoptosis has been reported in mouse, rat, rabbit, cow, and human cells (Szegezdi, 2003). Sustained endoplasmic reticulum stress leads to activation of GADD153 which can cause cell-cycle arrest and/or apoptosis. GADD153 is a proapoptotic transcription factor, ubiquitously expressed at low levels (Fornace, 1988; Park, 1992; Ron and Habener, 1992). GADD153 is present in the cytosol under normal conditions, and cellular stress leads to accumulation in the nucleus (Ron and Habener, 1992). Various physiological and pathological stimuli results in accumulation of misfolded proteins in the endoplasmic reticulum lumen resulting in endoplasmic reticulum stress (Harding, 1999). Strong or long-time

endoplasmic reticulum stress can result in apoptosis (Momoi, 2004). GADD153 is one of the highest inducible genes during endoplasmic reticulum stress (Bartlett, 1992; Okada, 2002; Oyadomari, 2001; Tang and Lane, 2000; Yang, 2005; Yoshida, 2000). Deficiency of GADD153 could protect cells from endoplasmic reticulum stress-induced apoptosis and overexpression of GADD153 can cause cell-cycle arrest and/or apoptosis, which indicates GADD153 plays an important role in the induction of endoplasmic reticulum stress-related apoptosis (van der Sanden, 2003).

Induction of GADD153 was shown to upset the cellular redox state by depletion of cellular GSH and exaggerated production of ROS (McCullough, 2001). Endoplasmic reticulum stress and disturbed calcium homeostasis is implicated in AD (Katayama, 2004; LaFerla, 2002; Verkhratsky, 2005). Oxidative stress, which may lead to endoplasmic reticular stress and eventually compromised endoplasmic reticulum stress response resulting RPE dysfunction are implicated in AMD (Ambati, 2005; Beatty, 2009; de Jong, 2006; Donoso, 2003). GADD153 induction correlates with the onset of apoptosis (Eymin, 1997; Friedman, 1996).

27-OHC significantly increased levels of GADD153 at both 10 μ M and 25 μ M concentrations (Figure 20B). The confocal microscopy imaging revealed an increase in the number of nuclei that are GADD153-positive on treatment with 25 μ M 27-OHC treatment (Figure 20C). The immunocytochemistry results confirmed the activation of GADD153 and its translocation into the nucleus.

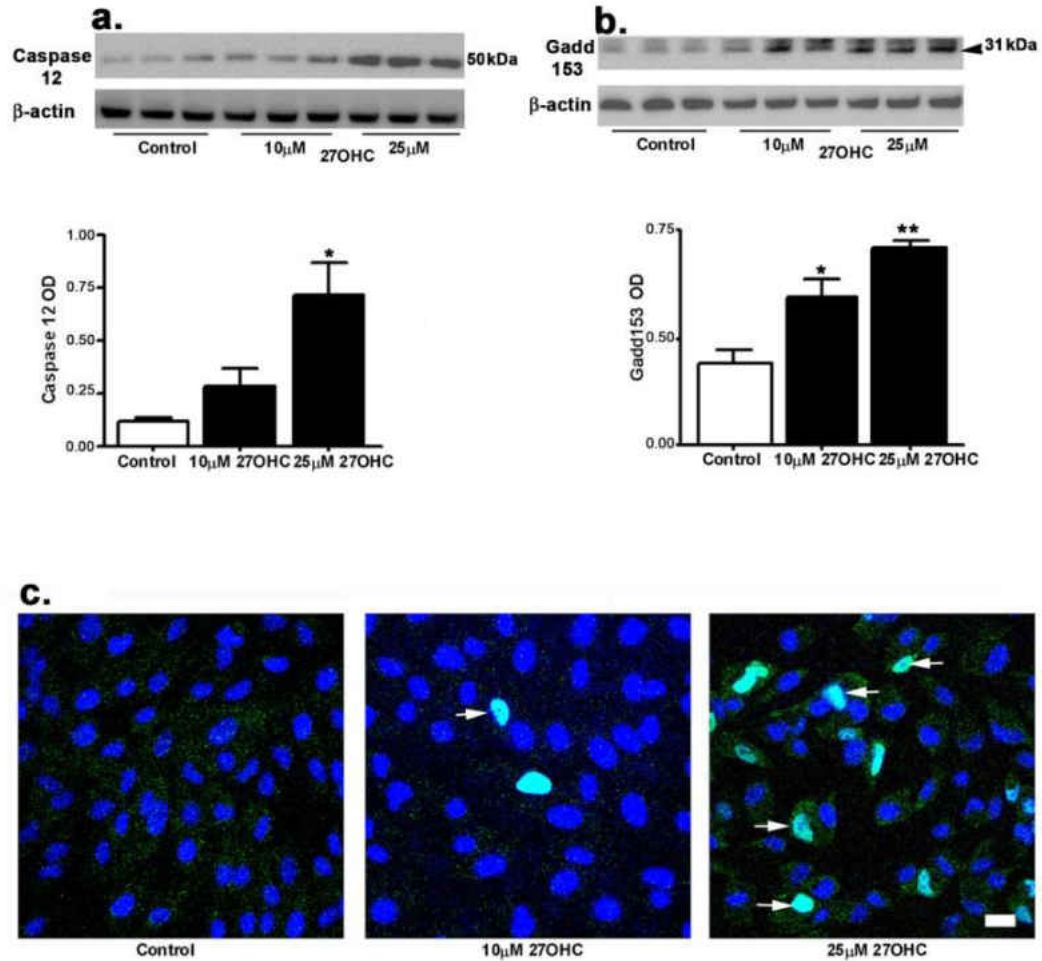


Figure 20: Endoplasmic reticulum stress induced by 27-OHC

A. Western blots showing an increase in levels of caspase 12 and B. GADD153, two specific markers of endoplasmic reticulum stress stress. C. Immunocytochemistry shows that GADD153 immunoreactivity (green) is localized in nucleus (DAPI, blue) following treatment with 27-OHC (arrows). * $p < 0.05$, ** $p < 0.01$ vs control; Bar, 20 μ m.

NF κ B-p65 subunit is a transcription factor, expressed in almost all cell types and is involved in processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. In normal conditions, NF- κ B is sequestered in the cytoplasm by inhibitor of kappa light polypeptide gene enhancer in B-cells (I κ B) proteins. One of the endoplasmic reticulum stress

response pathways involves activation of NF- κ B, for mediating immune and antiapoptotic responses. The stress leads to Ca²⁺ release from endoplasmic reticulum and reactive oxygen intermediates, which activate NF- κ B by degradation of I κ B. Free NF- κ B is transported into the nucleus, and induces transcription of target genes (Sen and Baltimore, 1986). Reactive oxygen intermediates (ROIs) can function as second messengers in NF- κ B activation (Meyer, 1993; Schmidt, 1995; Schreck, 1991). In neurons and astroglia of brain sections from AD patients, activation of NF- κ B was observed (Kaltschmidt, 1997). Aberrant activation of NF κ B is associated with macular degeneration along with many other diseases. NF- κ B can be activated by endoplasmic reticulum stress and can exert either cytoprotective or cytotoxic effects depending on the type of stimulus and duration (Pahl and Baeuerle, 1996).

In the present study, NF- κ B levels in the nucleus are significantly increased in the 27-OHC treated ARPE-19 cells (Figure 21B). The immunocytochemistry with antibody to NF- κ B further showed an emergence of nuclear staining for NF- κ B with 27-OHC treatment (Figure 21C). TNF- α is a multifunctional pro-inflammatory cytokine and is viewed as a classic regulator of cell death and is also activated by endoplasmic reticulum stress (Li, 2005). TNF- α is also an activator of NF- κ B. 27-OHC, at 25 μ M but not at 10 μ M, induces a significant increase in TNF- α levels as determined by an ELISA assay (Figure 21C).

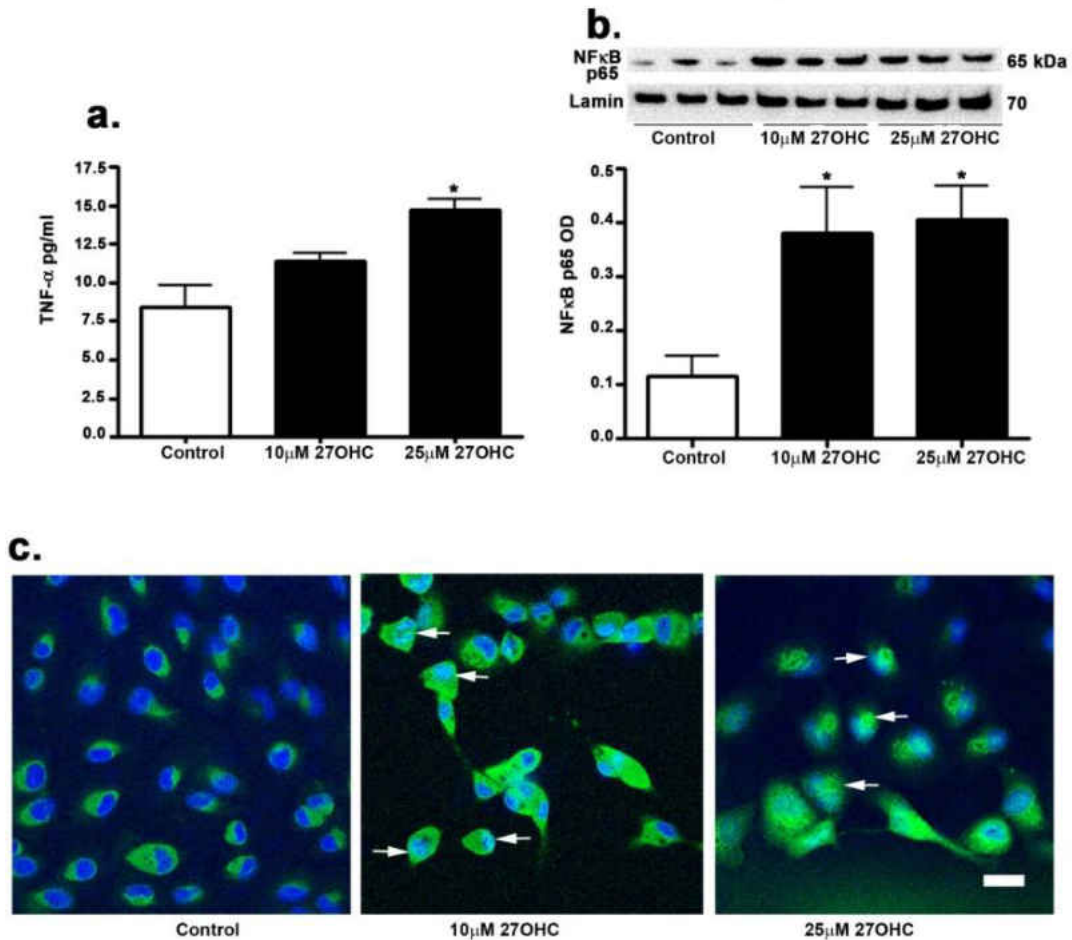


Figure 21: TNF- α and NF- κ B levels are increased by 27-OHC treatment

A. Treatment with 27-OHC increases TNF- α levels, as shown by ELISA and B. increased NF- κ B levels in the nucleus as shown by Western blot. C. Immunocytochemistry for NF- κ B shows increased immunoreactivity (green) and translocation into the nucleus (DAPI, blue) of this protein in the 27-OHC-treated cells (arrows, c). * $p < 0.05$ vs control; Bar, 20 μ m.

27-OHC-induced oxidative damage

Whether 27-OHC causes any oxidative stress on ARPE-19 cells was explored. The mechanistic basis of AMD disease is not clearly understood but may involve oxidative injury to retinal pigment epithelium. GSH, a tripeptide is very crucial molecule in cell defense against oxidative injury (Bok, 1985; Griffith,

1999; Kaplowitz, 1985; Meister and Anderson, 1983; Pompella, 2003; Sies, 1999). The GSH system, includes reduced GSH, GSSG and a number of related enzymes, is the main redox control system of the cell. GSH and its precursor amino acids protect against oxidative injury in cultured human RPE cells (Sternberg, 1993).

It is known that glutathione levels of a person with AMD are lower than of a person the same age with healthy eyes. An alteration in GSH levels gives assessment of toxicological responses and is an indicator of oxidative stress, potentially leading to apoptosis (Townsend, 2003). In this study oxysterol 27-OHC effect on glutathione levels in ARPE-19 cells was examined. Total GSH levels (reduced GSH plus GSSG) were quantified. GSH levels are significantly decreased in concentration dependent manner. 10 μ M and 25 μ M 27-OHC caused a significant reduction in glutathione (Figure 22A). These results suggest that 27-OHC reduced the anti-oxidant potential of cells, thereby increasing cell susceptibility to oxidative damage. Excessive production of ROS is known to lead to oxidative stress, loss of cell function, and ultimately to cell death.

This study showed that 27-OHC significantly increased heme oxygenase 1 (HO-1) levels in ARPE-19 cells (Figure 22C). HO-1 catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin, iron, and carbon monoxide (Tenhunen, 1968). HO-1 is mainly located in the endoplasmic reticulum, anchored by a single transmembrane domain. HO-1 is ubiquitously distributed and strongly induced by oxidative, endoplasmic reticulum, nitrosative, osmotic, and hemodynamic stress (Durante, 1997; Keyse, 1990; Liu, 2005; Motterlini, 1996; Tian, 2001; Wagner, 1997).

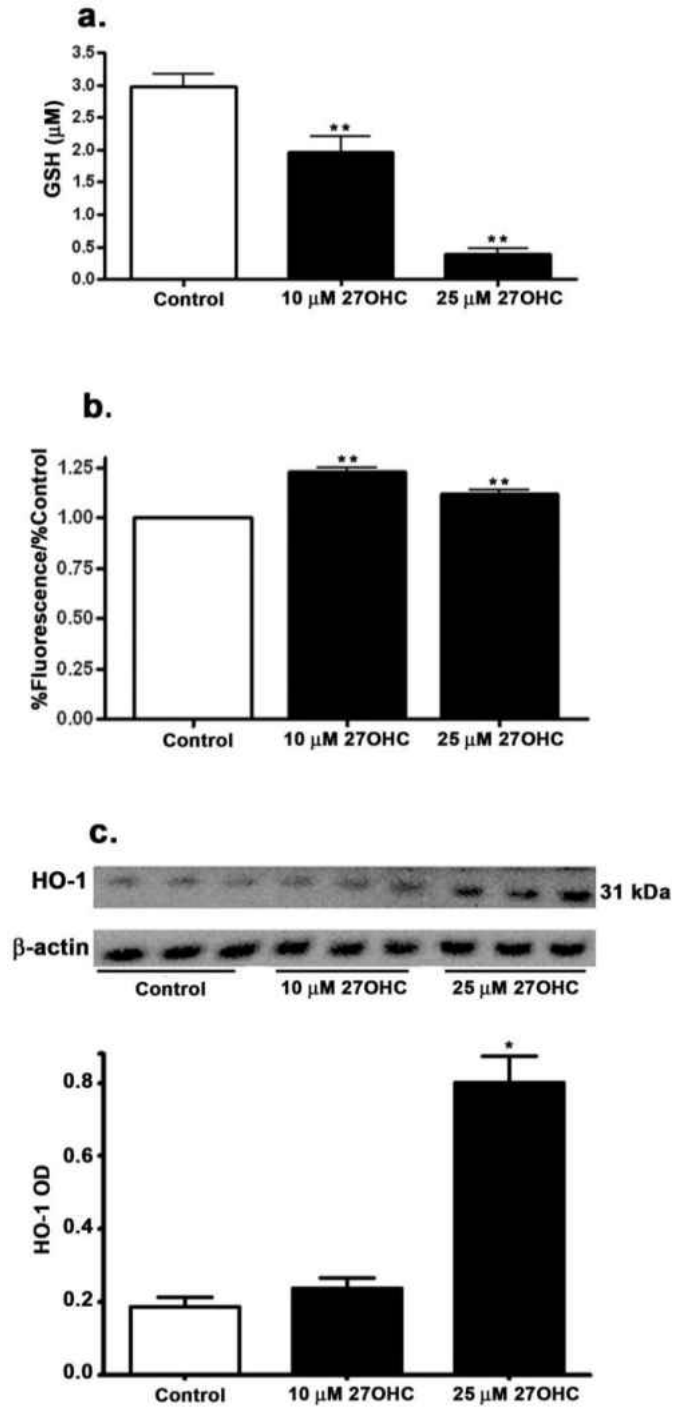


Figure 22: 27-OHC altered glutathione, ROS and HO-1 levels in ARPE-19 cells. A. Treatment with 27-OHC causes a decrease in glutathione concentrations. B. an increase in ROS generation as measured by DCFH-DA assay C. an increase in HO-1 levels (c). * $p < 0.05$, ** $p < 0.01$ vs control.

Induction of HO-1 by these biochemical stimuli provides an important cellular defense mechanism against tissue injury (Abrescia, 1979; Amersi, 1999; Clark, 2000; Foresti, 1999; Motterlini, 1996; Tian, 2001; Yet, 2001). HO-1 elevation is regarded as a general indicator of oxidative stress in cells and tissues (Applegate, 1991; Keyse, 1990; Stocker, 1990). GSH depletion may lead to enhanced HO-1 gene expression (Lautier, 1992). HO-1 immunoreactivity is significantly more in neurons of the AD patients temporal cortex and hippocampus than corresponding tissues derived from nondemented controls matched for age and postmortem interval (Schipper, 1995). HO-1 induction is a relatively early event in the pathogenesis of sporadic AD (Schipper, 2006). HO-1 presence at high levels is considered evidence that such stress is present. HO -1 and -2 are increased in RPE of AMD-affected maculas (Frank, 1998).

27-OHC triggered cell death in ARPE-19 cells

LDH is released into the surrounding medium from cells when they lose membrane integrity. After 24 h treatment with 27-OHC, cell viability was quantitatively determined by the measurement of lactate dehydrogenase (LDH) released from cells into the medium by using the CytoTox-ONE homogeneous membrane integrity assay. This fluorometric assay estimates the number of nonviable cells by measuring the release of LDH from cells with a damaged membrane. The amount of fluorescence produced is proportional to the number of lysed cells. 27-OHC treatment leads to a significant increase in the number of dead cells (Figure 23A). Cell death involves various events including the loss of mitochondrial membrane potential (Green and Reed, 1998).

The fluorescent cationic dye JC-1 was used, which signals the loss of mitochondrial membrane potential (Smiley, 1991). The ratio of red (monomeric form of JC-1) to green fluorescence (aggregates of JC-1) was determined. 10 μ M and 25 μ M 27-OHC reduced the red: green fluorescence ratio, indicating a significant decrease in the mitochondrial membrane potential (Figure 23B).

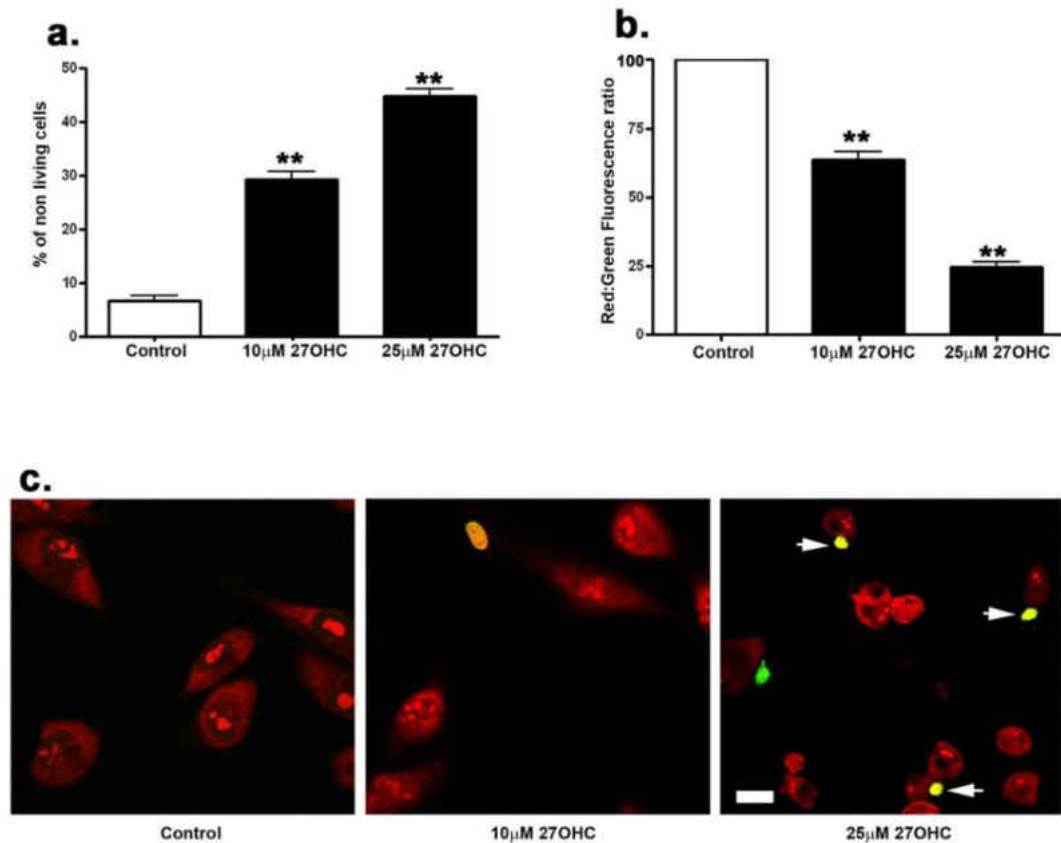


Figure 23: 27-OHC is deleterious to ARPE-19 cells.

A. Cyto Tox-ONE homogenous membrane integrity assay shows that 27-OHC is cytotoxic to cells B. and decreases the mitochondrial membrane potential as measured by a reduction in red (non-apoptotic cells)/green (apoptotic cells) fluorescence. C. TUNEL assay shows that 27-OHC increases the number of apoptotic cells (arrow) with 25 μ M 27-OHC. Apoptosis is evidenced by DNA fragmentation labeled with fluorescein (green); Propidium iodide (red) is used to label cell nuclei. **p < 0.01 vs control, Bar, 20 μ m.

TUNEL assay, used to detect apoptotic cell death, showed no apoptotic cells in untreated cells; in the 27-OHC-treated cells, TUNEL-positive cells were observed with a number that is higher with 25 μ M than 10 μ M 27-OHC (Figure 23C). All together, these results demonstrate that 27-OHC compromises cell survival. 25-OHC and 7-KC is shown to be cytotoxic to cultured RPE and retinal cells (Ong, 2003). Presence of 7-KC in lipid deposits from primate retina suggested oxysterol involvement in AMD development (Moreira, 2009). 7-KC treated ARPE-19 cells showed some of the characteristics of apoptosis (Luthra, 2006). Cytotoxicity of oxLDL on RPE cells was shown to be dependent on 7-KC formation (Rodriguez, 2004). 7-KC and 25-OHC have been demonstrated to have cytotoxic, pro-oxidative, and/or angiogenic activities on ARPE-19 cells.

27-OHC is ligand for both ER and LXR. 27-OHC has both agonist and antagonist activities depending on cell type. For example 27-OHC displays agonist activity in breast cancer cells and antagonistic activity in cardiovascular system. 25-OHC is also a ligand for both ER and LXR. 7-KC has been described as an LXR agonist (Janowski, 1999). LXR involvement is suggested in 7-KC mediated induction of VEGF expression (Moreira, 2009).

ER α and ER β , are expressed in ARPE-19 cells

The ER α and β are ligand-inducible transcription factors and belong to the nuclear receptor superfamily. The two estrogen receptor subtypes-ER α and ER β are shown to be expressed in the male and female retina (Munaut, 2001; Ogueta, 1999). ERs existence in ARPE-19 cells was probed by Western blotting (Figure 24). Triplicate cell lysates were resolved by SDS-PAGE and investigated for ER α and ER β expression. ER α band was detected approximately at 68 kDa,

and ER β band was detected at approximately at 53 kDa. Both cytosolic and nuclear fractions were used for probing of ER α and ER β . For cytosolic fractions, β -actin was used as loading control, and for nuclear fractions, lamin A was used as loading control. ER α and ER β expression in these cells was proven by this study.

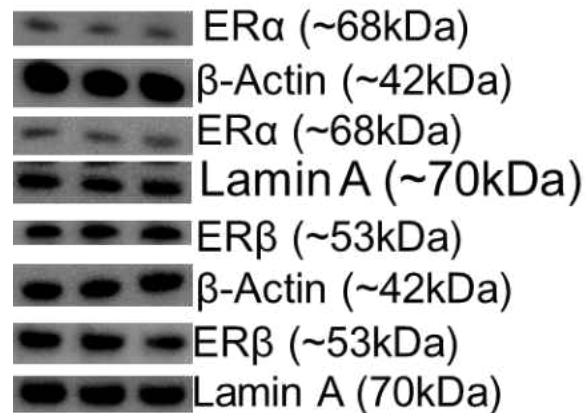


Figure 24: ARPE-19 cells express ER α and ER β

Western blots showing ER α and ER β receptor protein expression in ARPE-19 cells. Triplicate cell lysates were resolved by SDS-PAGE and probed for ER α and ER β . β -actin and lamin were used as loading controls for cytosolic and nuclear fractions respectively.

ERE-luciferase activity is increased by ER agonist E2

The binding of E2 to ERs induce receptor activation, which lead to interaction with specific EREs located within the regulatory regions of target genes. Transfection of ERE-luciferase construct and luciferase activity measured by dual luciferase assay elucidated functional activity of ER. 10nM E2, an ER agonist caused an increase in ERE-luciferase activity denoted by ERE-relative luciferase units, when compared to vehicle treatment confirming ER functionality (Figure 25).

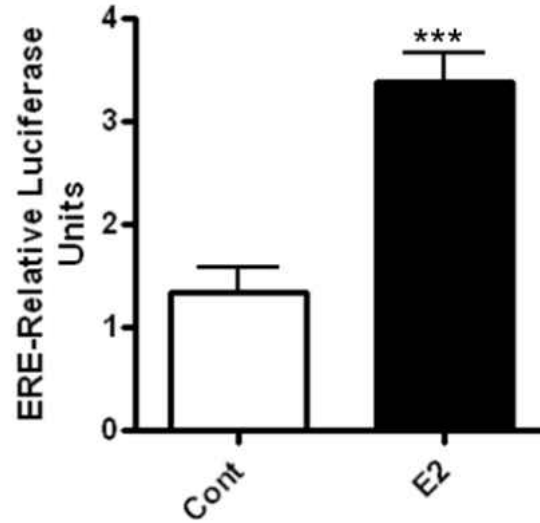


Figure 25: E2 increased ERE-luciferase activity

ARPE-19 cells were transfected in parallel with ERE-reporter and negative control. Transfected cells were subjected to 10 nM E2 treatment for 24 h. Cells were lysed and dual luciferase assay was performed, and reporter activity values are expressed as arbitrary units; *** $p < 0.001$ vs control

LXR α and LXR β are expressed in ARPE-19 cells

mRNA for LXRs were detected in ARPE-19 cell culture, primary RPE culture and freshly isolated RPE cells (Dwyer, 2011; Ishida, 2004). Unpublished observations by Rodriguez group noted LXRs expression in retina (Rodriguez and Larrayoz, 2010). There were no published studies showing LXR α and LXR β expression. Expression of LXRs in ARPE-19 cells was researched in this study. Triplicate cell lysates were resolved by SDS-PAGE and probed for LXR α and LXR β (Figure 26). LXR α band was detected approximately at 50 kDa, and LXR β band was detected at approximately at 55 kDa. Both cytosolic and nuclear fractions were used for probing of LXR α and LXR β . For cytosolic fractions, β -actin was used as loading control, and for nuclear fractions, lamin A was used as

loading control. Western blot analysis of cytoplasmic and nuclear extracts showed LXR α and LXR β expression (Figure 26).

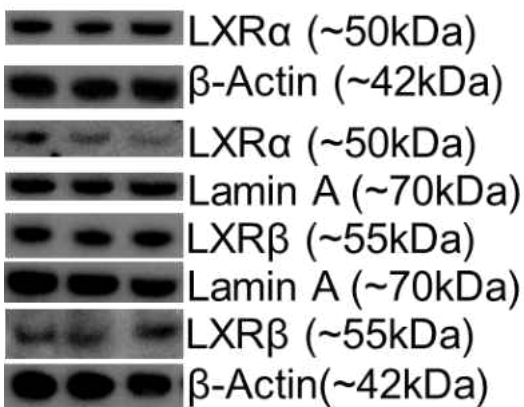


Figure 26: ARPE-19 cells express LXR α and LXR β

Western blots showing LXR α and LXR β receptor protein expression in ARPE-19 cells. Triplicate cell lysates were resolved by SDS-PAGE and probed for LXR α and LXR β . β -actin and lamin were used as loading controls for cytosolic and nuclear fractions respectively.

LXRE-luciferase activity is increased by LXR agonist GW3965

Western blot results confirmed the existence of LXR α and LXR β receptors. However, whether these receptors are transcriptionally active in ARPE-19 cells is unknown. Transfection of LXRE-luciferase construct in parallel with negative control construct and dual luciferase assay upon treatment with LXR agonist GW3965 elucidated functional activity of LXR. GW3965 caused an increase in LXRE-luciferase activity denoted by LXRE-relative luciferase units, when compared to vehicle treatment confirming LXR functionality (Figure 27). There is endogenous LXR transcriptional activity in these cells, as shown by control cells treated with vehicle also exhibiting LXR-luciferase activity.

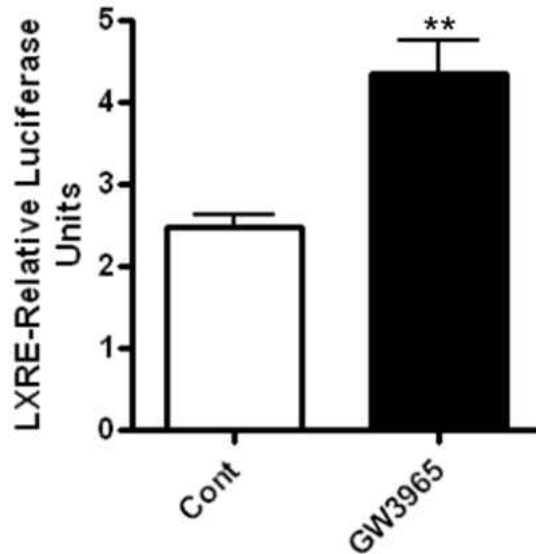


Figure 27: GW3965 increased LXRE-luciferase activity

ARPE-19 cells were transfected in parallel with LXRE reporter and negative control. Transfected cells were subjected to LXR agonist GW3965 treatment. After 24 h Dual luciferase assay was performed, and reporter activity values are expressed as arbitrary units. GW3965 increased LXRE-luciferase activity indicating that LXR are functional.

**p<0.01

25-OHC, 27-OHC and 7-KC inhibited ER mediated transcriptional activity

It is unknown whether oxysterols, 27-OHC, 25-OHC and 7-KC have any effect on ER mediated transcription in ARPE-19 cells. Therefore in the present study 27-OHC, 25-OHC and 7-KC influence, if there is any, on ER mediated transcriptional activity in retinal pigment epithelial cells was investigated.

ERE-luciferase reporter transfected ARPE-19 cells were treated with 27-OHC, 25-OHC and 7-KC for 24 h. Dual luciferase assay upon treatment revealed that all three oxysterols significantly reduced ERE-luciferase activity, indicating suppressive effects of oxysterols on estrogen transcriptional response element mediated transcription (Figure 28).

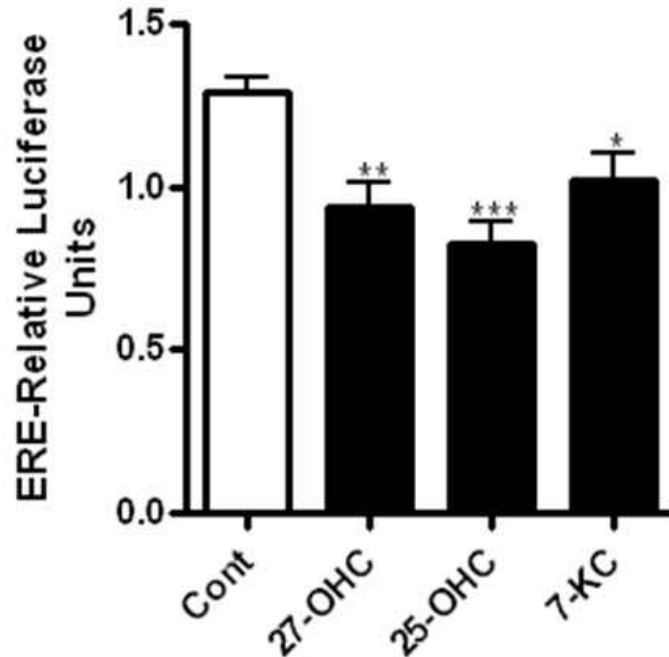


Figure 28: 27-OHC, 25-OHC and 7-KC decreased ERE-luciferase activity

ARPE-19 cells were transfected in parallel with ERE reporter, which is a mixture of inducible ERE-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct and negative control, which is a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct. Transfected cells were subjected to oxysterol treatments for 24 h. Dual luciferase assay was performed, and reporter activity values are expressed as arbitrary units. 27-OHC, 25-OHC and 7-KC decreased ERE-luciferase activity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

25-OHC and 27-OHC increased LXR mediated transcriptional activity

25-OHC and 27-OHC were shown to be natural endogenous ligands for LXRs in several cell types. It is unknown whether these oxysterols have any influence on LXR-mediated transcriptional activity in RPE cells. LXR-mediated transcriptional activity was examined in ARPE-19 cells upon treatment with 27-OHC, 25-OHC and 7-KC. Transfection of LXRE-luciferase construct and

luciferase activity measured by dual luciferase assay upon oxysterols 27-OHC, 25-OHC and 7-KC treatments elucidated LXR activation in presence of 25-OHC and 27-OHC. 7-KC did not cause any significant change in LXRE-luciferase activity when compared with vehicle treatment.

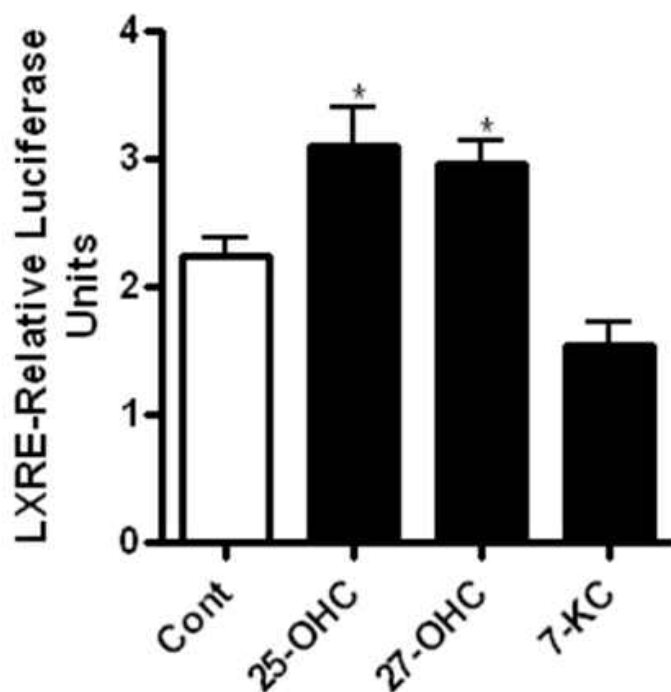


Figure 29: 25-OHC and 27-OHC increased LXR mediated transcriptional activity. ARPE-19 cells were transfected in parallel with LXRE reporter, which is a mixture of inducible LXRE-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct and negative control, which is a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct. Transfected cells were subjected to oxysterol treatments for 24 h. Dual luciferase assay was performed, and reporter activity values are expressed as arbitrary units. 27-OHC, 25-OHC and 7-KC significantly decreased LXRE-luciferase activity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

ER agonist E2 protected cells from 25-OHC and 27-OHC induced cytotoxicity

27-OHC is shown to be cytotoxic to ARPE-19 cells (Dasari, 2010). 25-OHC and 7-KC were also shown to be cytotoxic oxysterols in ARPE-19 cells and other cell types (Luthra, 2006; Rodriguez, 2004; Dugas, 2010). E2 has been suggested as a potential therapeutic agent in the management of several neurodegenerative disorders. E2 rescued undifferentiated neuroblastoma cells from 24-OHC induced neurotoxicity (Kolsch, 2001). E2 treatment was reported to attenuate release of cytokines, reactive oxygen species, and complement proteins (Bruce-Keller, 2000; Drew and Chavis, 2000).

Whether preincubation with E2 offers any cytoprotection in 25-OHC, 27-OHC and 7-KC induced cytotoxicity in ARPE-19 cells was examined (Figure 30). ARPE-19 cells were treated with oxysterols for 24 h in the presence or absence of 10 nM E2, an ER agonist and 1 μ M ICI, an antagonist. After 24 h incubation, cell viability was quantitatively determined by the measurement of LDH released from cells into the medium by using the CytoTox-ONE homogeneous membrane integrity assay. 10 μ M 25-OHC, 10 μ M 27-OHC and 25 μ M 7-KC significantly induced cytotoxicity. 25-OHC and 27-OHC induced cytotoxicity was significantly reduced in cells treated with E2 (Figure 30A, B). But 7-KC induced cytotoxicity was not reversed by E2 (Figure 30 C). ER antagonist ICI did not offer any cytoprotection. E2 and ICI treatments alone are comparable with control cells. ICI was added 30 min before the addition of E2, and E2 was added 2 h before oxysterols addition to cells.

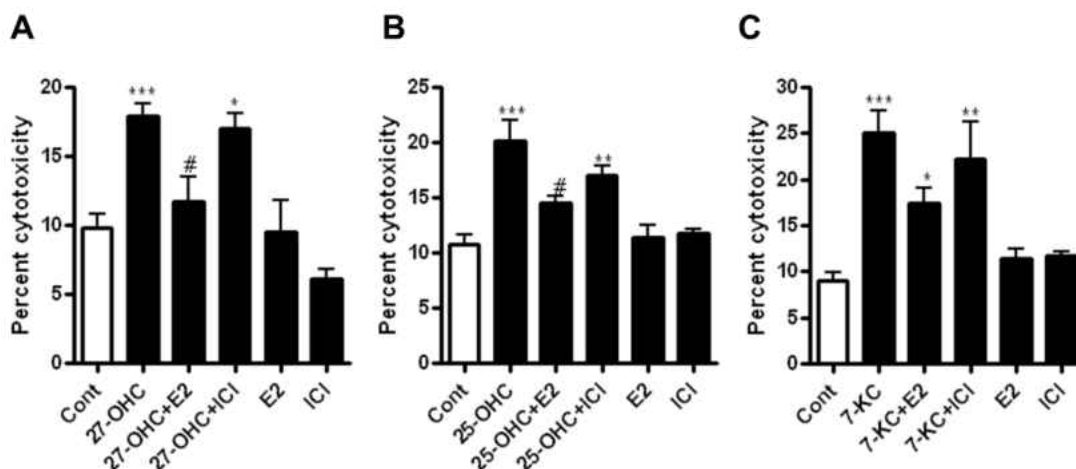


Figure 30: E2 protected cells from 25-OHC and 27-OHC induced cytotoxicity

CytoTox-ONE homogenous membrane integrity assay was used for measuring cytotoxicity. A. 27-OHC is cytotoxic to cells, when cells were treated with E2, cytotoxicity induced by 27-OHC is reduced. ICI did not protect cells from 27-OHC induced cytotoxicity. B. 25-OHC is also cytotoxic to cells. When cells were treated with E2, 25-OHC induced cytotoxicity was reduced, whereas ICI is not protective. C. 7-KC is cytotoxic to cells and neither E2 nor ICI protected cells from 7-KC induced cytotoxicity. * $p < 0.05$, *** $p < 0.001$ vs control; # $p < 0.05$ vs oxysterol.

Since 25-OHC, 27-OHC and 7-KC were shown to be ligands of LXRs in various cell types, whether LXR agonist GW3965 or antagonist ECHS offer any beneficial role in oxysterol induced cytotoxicity was then investigated.

After 24 h incubation with 10 μM 25-OHC, 10 μM 27-OHC and 25 μM 7-KC in the presence or absence of 1 μM GW3965 and 1 μM ECHS, cell viability was quantitatively determined by the measurement of LDH released from cells into the medium by using the CytoTox-ONE homogeneous membrane integrity assay. Neither LXR agonist, GW3965, nor LXR antagonist ECHS reduced oxysterols 27-

OHC, 25-OHC and 7-KC induced cytotoxicity in ARPE-19 cells. LXR agonist GW3965 and LXR antagonist ECHS themselves did not cause cytotoxicity.

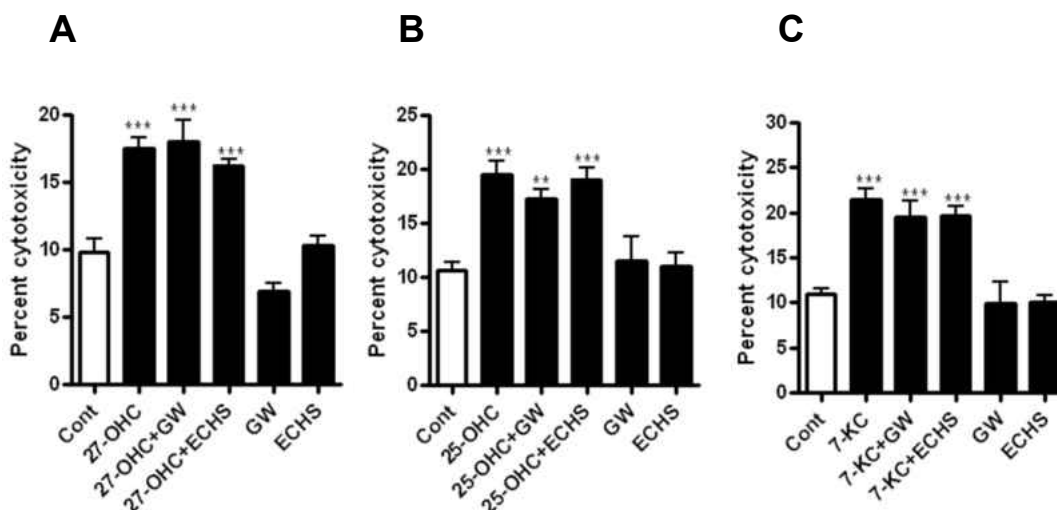


Figure 31: GW and ECHS did not offer cytoprotection against 25-OHC and 27-OHC induced cytotoxicity

CytoTox-ONE homogenous membrane integrity assay was used for measuring cytotoxicity. A. Treatment of GW3965 or ECHS along with 27-OHC did not offer any protection against 27-OHC induced cytotoxicity. B. Treatment of GW3965 or ECHS along with 25-OHC did not offer any protection against 25-OHC induced cytotoxicity. C. Treatment of GW3965 or ECHS along with 7-KC did not offer any protection against 7-KC induced cytotoxicity. *p < 0.05, ***p < 0.001 vs control

E2 counteracted 27-OHC and 25-OHC induced mitochondrial membrane potential decline

Cell death comprises several events including the loss of mitochondrial membrane potential (Green and Reed, 1998). 27-OHC is shown to significantly decrease mitochondrial membrane potential in ARPE-19 cells (Figure 23B, 32A). 25-OHC and 7-KC also significantly reduced the red to green fluorescence ratio, indicating a decrease in the mitochondrial membrane potential (Figure 32B, C).

ARPE-19 cells were treated with 10 μ M 25-OHC, 10 μ M 27-OHC and 25 μ M 7-KC for 24 h in the presence or absence of 10 nM E2, an ER agonist and 1 μ M ICI, an antagonist of ER. Treating cells with E2 counteracted 27-OHC and 25-OHC induced mitochondrial membrane potential decline (Figure 32A, B), whereas E2 treatment could not protect cells from 7-KC induced mitochondrial membrane potential decline (Figure 32C). Treating cells with ER antagonist ICI did not offer any protection against mitochondrial membrane potential decline (Figure 32A, B, C).

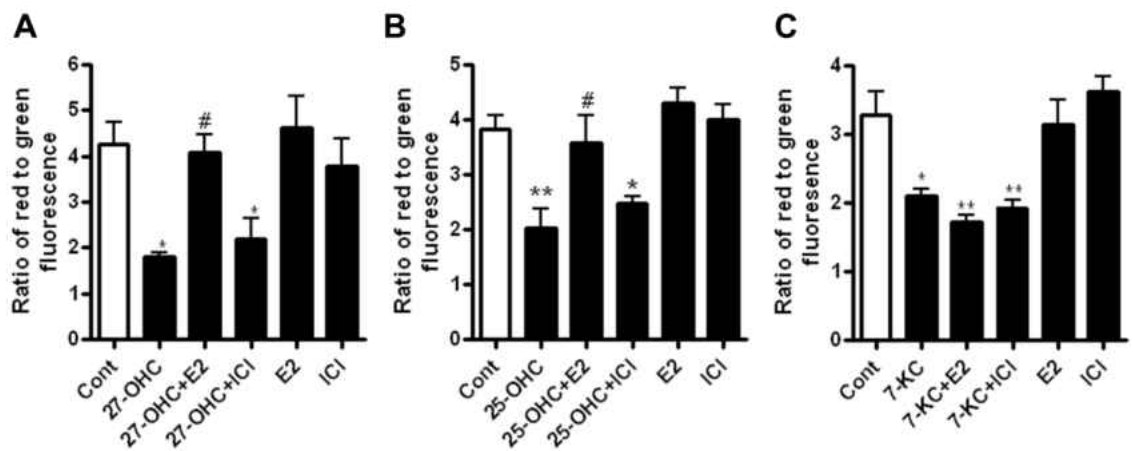


Figure 32: E2 counteracted 27-OHC and 25-OHC induced mitochondrial membrane potential

Mitochondrial membrane potential was measured by JC-1 assay. A. 27-OHC decreases the mitochondrial membrane potential as shown by a reduction in red (non-apoptotic cells)/green (apoptotic cells) fluorescence. E2 counteracted the mitochondrial membrane potential decline, whereas ICI did not. B. 25-OHC caused a decrease in mitochondrial membrane potential decrease, which is counteracted by E2 but not by ICI. C. 7-KC induced mitochondrial membrane potential decline was not counteracted by E2 or ICI; * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$ vs oxysterols.

And then, whether LXR agonist GW3965 and antagonist ECHS has any protective role in mitochondrial membrane potential was investigated in ARPE-19 cells. After 24 h incubation with 10 μ M 25-OHC, 10 μ M 27-OHC and 25 μ M 7-KC in the presence or absence of 1 μ M GW3965 and 1 μ M ECHS, mitochondrial membrane potential was measured by JC-1 assay. Neither GW3965 nor ECHS protected cells from oxysterols 27-OHC, 25-OHC and 7-KC induced mitochondrial membrane potential decline. Either GW3965 or ECHS alone did not significantly change mitochondrial membrane potential.

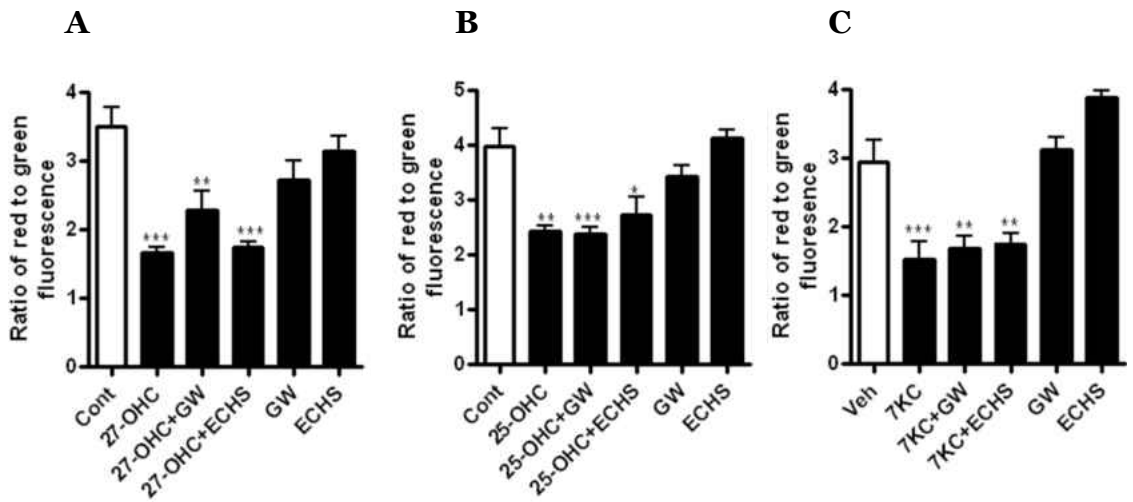


Figure 33: GW3965 and ECHS did not offer any protection against 25-OHC, 27-OHC and 7-KC induced mitochondrial membrane potential decline

Mitochondrial membrane potential was measured by JC-1 assay. 25-OHC, 27-OHC and 7-KC reduced mitochondrial membrane potential. A. Neither GW3965, nor ECHS counteracted 27-OHC induced mitochondrial membrane potential decline. B. Neither GW3965, nor ECHS counteracted 25-OHC induced mitochondrial membrane potential decline. C. Neither GW3965, nor ECHS counteracted 7-KC induced mitochondrial membrane potential decline

E2 treatment protected cells from 27-OHC and 25-OHC induced Apoptosis

The pathogenesis involves apoptosis of the RPE followed by death of the underlying photoreceptors (Hinton, 1998; Xu, 1996). Due to RPE's unique location and role, it is an ideal environment for the accumulation of reactive ROIs (Beatty, 2009) which in turn leads to RPE death (Liang and Godley, 2003). 27-OHC reported to cause apoptosis in ARPE-19 cells (Figure 23C).

ARPE-19 cells were treated with 10 μ M 25-OHC, 10 μ M 27-OHC and 25 μ M 7-KC for 24 h in the presence or absence of 10 nM E2, an ER agonist and 1 μ M ICI, an antagonist of ER. and in the presence or absence of 1 μ M GW3965, an LXR agonist and 1 μ M ECHS, an LXR antagonist. ICI was added 30 min before the addition of E2, and E2 was added 2 h before oxysterols addition to cells. ECHS was added 30 min before the addition of GW3965, and GW3965 was added to 2 h before oxysterol addition to cells. After 24 h of incubation, apoptosis was detected in ARPE-19 cells by TUNEL assay, which detects fragmented DNA. TUNEL staining showed no apoptotic cells in the control cells whereas 27-OHC, 25-OHC and 7-KC induced apoptotic cell death which was shown by green staining. Red color is a counterstain for showing nucleus. Treating cells with E2 counteracted from oxysterol induced apoptosis. ICI, GW3965 and ECHS did not offer any protection against apoptosis induced by oxysterols. E2, ICI, GW3965 and ECHS themselves did not induce apoptosis.

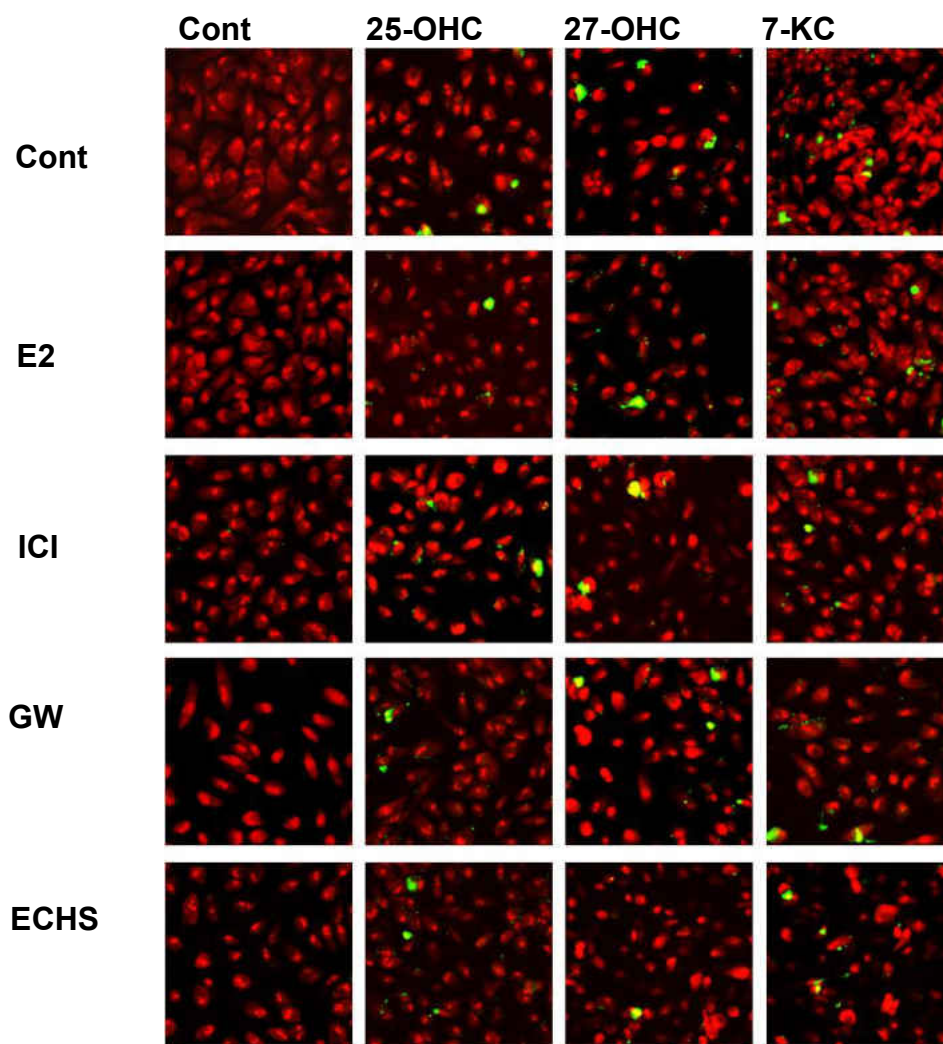


Figure 34: E2 treatment protected cells from 27-OHC and 25-OHC induced apoptosis

TUNEL assay shows that 27-OHC, 25-OHC and 7-KC increases the number of apoptotic cells. E2 treatment reduced apoptosis induced by 25-OHC and 27-OHC. E2 did not rescue cells from 7-KC induced cytotoxicity. ICI, GW3965 and ECHS did not offer any protection against oxysterol induced apoptosis. Apoptosis is evidenced by DNA fragmentation labeled with fluorescein (green); Propidium iodide (red) is used to label cell nuclei.

CHAPTER IV

DISCUSSION

Cholesterol dyshomeostasis has been implicated in the pathogenesis of AD (Wellington, 2004) and high-fat diet rich in saturated fatty acids and cholesterol is suggested to be associated with AMD (Mares-Perlman, 1995). Epidemiological studies showed that increased plasma cholesterol levels are associated with atherosclerosis and increased risk of dementia, including AD (Kivipelto and Solomon, 2006; Solomon, 2009). Although, there is no consensus on the association of plasma lipid levels and AMD, two recent genome wide association studies implicated cholesterol metabolism involvement in AMD (Chen, 2010; Neale, 2010).

Strong evidence linking AD and retinal degeneration (Guo, 2010) incited this study. The retina is an extension of the brain, and has the potential to reflect AD brain pathology. Extracellular A β deposition, oxidative stress, and inflammation are implicated in both AD and AMD (Butterfield, 2002; Ding, 2009). Ganglion cell death has been recently documented in retinas of AD mouse models (Cordeiro, 2010). AMD is characterized by the abnormal, extracellular deposits of cellular debris called drusen that are located between Bruch's membrane and the RPE. Drusen contain various components including A β (Bressler, 1990; Dentchev, 2003). Amyloid misfolding-inducing inflammation has been suggested to mediate retinal neurodegeneration (Berisha, 2007). The

extent to which the cholesterol-fed rabbit model for AD also exhibits retinal pathology relevant to AMD was investigated. Cholesterol-enriched diet increased A β levels and oxidative stress, induces apoptotic cell death and morphological changes, and alters cholesterol metabolism in rabbit retinas. A β steady state levels are determined by the balance between its production and removal levels. A β is generated from APP and degraded by several enzymes. Initial cleavage of APP involves BACE-1 enzyme to yield A β . A β ₁₋₄₀ and A β ₁₋₄₂ are then degraded by various enzymes including IDE.

Feeding rabbits a diet rich in cholesterol increased A β peptide levels as well as levels of BACE-1. These results suggest that increased A β levels in rabbit retinas are derived from an increased APP processing by BACE-1. It may also be possible that increased A β transport from the circulation to retinas contributes to the elevated levels of A β peptide in retinas. While some studies showed an increase in IDE in AD patients (Caccamo, 2005; Dorfman, 2010; Miners, 2009; Vepsalainen, 2008), others have showed reduction in enzyme levels (Cook, 2003; Perez, 2000). In one of these studies, it was suggested that the reduction in IDE activity is not the primary cause of A β accumulation but rather is a late stage phenomenon secondary to neurodegeneration (Caccamo, 2005).

Retinal IDE levels were increased in the cholesterol-fed rabbits. The increase in IDE levels may have been a mechanism for coping up with the A β overload. As A β is a neurotoxic peptide and its accumulation in the retina may promote oxidative damage and cell death, the extent to which accumulation of A β peptide is associated with increased ROS and apoptotic cell death was explored.

Cholesterol-enriched diet-induced A β accumulation is associated with increased oxidative stress. Proteins, carbohydrates, membrane lipids and nucleic acids are all vulnerable to ROS damage, and this damage is believed to contribute to the pathogenesis of many diseases. Oxidative injury to cells is associated with several diseases, including AD (Aksenov, 2001) and AMD (AREDS report 9, 2001). DCFH-DA assay indicated that there is an increase in ROS in the retinas of cholesterol-fed rabbits. Hydrogen peroxide, which is also considered as reactive oxygen species, was increased and peroxidase activity is decreased in cholesterol-fed rabbit retinas.

HO-1 is a sensor of oxidative stress that degrades heme, leading to formation of biliverdin and carbon monoxide (Maines, 1988; Tenhunen, 1968). HO-1 has been shown to be increased in RPE of AMD-affected maculas (Frank, 1998). HO-1 induction is suggested to be an early event in the pathogenesis of sporadic AD (Schipper, 2006) and has been demonstrated to be closely associated with neurofibrillary pathology in AD (Smith, 1994). We have previously shown that cholesterol-fed rabbits exhibit increased levels of ROS and HO-1 in addition to increased A β in the brain (Ghribi, 2006). This study showed that HO-1 levels are also increased in the retinas of cholesterol-fed rabbits.

Pathogenesis of many retinal and ocular diseases involves apoptosis. Histopathological studies suggest that the retinal pigment epithelium damage occurs first, followed by death of photoreceptors (Sarks, 1988), with rod photoreceptor cell loss preceding that of cone photoreceptor cells (Curcio, 1996; Curcio, 2001). Characteristic DNA fragmentation of apoptosis was detected in photoreceptors in 4 of 16 eyes with AMD (Xu, 1996). It was shown that apoptosis

is the cause of photoreceptor cell death in three mouse models of Retinitis pigmentosa (Portera-Cailliau, 1994). It was also shown that photic exposure triggers apoptosis of photoreceptor cells (Abler, 1996). Retinal ganglion cells also undergo apoptosis in glaucoma (Kerrigan, 1997; Quigley, 1995). As apoptosis may be involved in AMD and other retinal diseases and cholesterol-enriched diet has been shown to induce apoptosis in rabbit brains (Ghribi, 2006), the extent to which cholesterol induces apoptotic cell death in retinas was inspected here.

The Bcl-2 family of proteins includes both pro- and anti-apoptotic members. Bcl-2 is the most prominent anti-apoptotic member and is an important regulator of photoreceptor cell death in retinal degenerations (Chen, 1996). Bcl-2 has been shown to reduce apoptosis by facilitating recovery of mitochondrial DNA damage (Deng, 1999). Anti-apoptotic protein Bcl-2 levels were decreased and the levels of pro-apoptotic protein Bax were increased in retinas of cholesterol-fed rabbits. Cholesterol-enriched diet also increased levels of GADD153, a protein that induces cell death and upsets the cellular redox state by depleting cellular glutathione and exaggerating the production of ROS (McCullough, 2001).

Development of drusen is one of the earliest clinical features of AMD. Drusen are extracellular deposits of lipids, proteins, glycoproteins, and cellular debris that accumulate between collagenous layer of Bruch's membrane and basal lamina of the RPE. Recent studies found many drusen constituents which include cholesterol, apolipoproteins, and complement components (Crabb, 2002; Malek, 2003). In this study, debris-like material between RPE and choroid capillaries in retinas of cholesterol-fed rabbits was detected. The debris-like

material known as drusen is regarded as a hallmark feature of AMD. In addition, GFAP is strongly upregulated in glial cells in response to neuronal damage and is best known marker for gliosis. Retinal macroglial cells (astrocytes and Müller cells) have an active role in normal retinal function (Newman and Reichenbach, 1996) as well as in pathology (Dyer and Cepko, 2000). Upregulation of GFAP expression, an indicator of reactive gliosis, has been demonstrated in response to various retinal pathologies including mechanical injury (Dyer and Cepko, 2000), retinal detachment (Okada, 1990), diabetic retinopathy (Mizutani, 1998), glaucoma (Wang, 2000), retinal ischaemia (Nishiyama, 2000), and photoreceptor degeneration (Eisenfeld, 1984). Increased GFAP expression in macroglia has also been described in retinas with AMD (Guidry, 2002; Madigan, 1994; Ramirez, 2001). The present study shows astrogliosis in the retinas of cholesterol-fed rabbits as shown by GFAP immunostaining.

Vitronectin has regulatory roles in inflammation and phagocytosis (Preissner and Seiffert, 1998). Increased vitronectin deposition in retina is suggested to participate in the pathogenesis of AMD (Hageman, 1999). A marked increase in vitronectin immunoreactivity in retinas from cholesterol-fed rabbits was observed. All together, the data revealed that cholesterol-enriched diets cause structural and morphological changes relevant to AMD. However, the mechanisms by which dietary cholesterol cause these changes in retinas are not well known. Recent genome wide association studies implicated cholesterol metabolism involvement in AMD (Chen, 2010; Neale, 2010). Cholesterol is constantly taken up by retina via LDL receptors from the circulation in addition to endogenous cholesterol synthesis.

Cholesterol-enriched diet caused an accumulation of cholesterol in the retina. As excess cholesterol in cells is detrimental, various mechanisms are necessary for cholesterol efflux from cells. These mechanisms include passive diffusion, oxysterols formation, and reverse cholesterol transport. ABCA-1 has been shown to play a role in the transport of cholesterol, and was detected in the retina of various organisms (Duncan, 2009; Tserentsoodol, 2006a).

ABCA-1 levels are increased in the cholesterol-fed rabbits, implying an increase in the cholesterol content in cells and efflux through ABCA-1. Cholesterol can be oxidized by enzymatic and non-enzymatic pathways. One of the most important enzymatically generated side-chain oxysterols is 27-OHC. This oxysterol is formed from cholesterol by CYP27A1 (Bjorkhem, 2009). CYP27A1 expression was increased in retinas of cholesterol-fed rabbits as shown by laser scanning confocal microscopy and Western blotting. 27-OHC and other oxysterols concentrations were measured in retinas by mass spectrometry. 27-OHC, 22-OHC and 24-OHC levels were increased in cholesterol-fed rabbit retinas providing evidence that elimination of excess cholesterol to oxysterols takes place in the retina. In contrast to these results in rabbit, a recent study could not detect 27-OHC in the bovine and human retinas, but found that its oxidation product, 5-cholestenoic acid is the most abundant oxysterol (Mast, 2011). 5-cholestenoic acid was not measured in the present study.

Other enzymatically generated oxysterols including 7 α -OHC, 4 β -OHC and 25-OHC catalyzed by CYP7A1, CYP3A4 and cholesterol 25-hydroxylase respectively were also measured. These enzymes oxidize cholesterol for various purposes including cholesterol elimination. Even though none of these enzymes

were reported to be expressed in the retina, their oxysterol products were found in retinas, most probably coming from blood circulation as retina has access to blood borne lipids (Fliesler and Bretillon, 2010). These findings suggest an increase in cholesterol in retinas as well as an increase in cholesterol metabolites. Numerous studies suggested cytotoxic effects of oxysterols are associated with human diseases including AD and AMD (Vejux, 2008). Increased oxysterol concentrations subsequent to increased cholesterol levels may contribute to the generation of AMD-like pathological hallmarks.

It is known that deficiency in 7- α -hydroxylase, an enzyme needed to prevent 27-OHC accumulation, causes severe neonatal cholestatic liver disease, where 27-OHC concentrations in the plasma could reach up to 800 μ M (Setchell, 1998). Similar abnormal accumulation of these metabolites in the retina may put this tissue in danger of degeneration. Of these oxysterols, 7-KC has been suggested to play a key role in the pathogenesis of AMD and has been recently shown to induce DNA damage in human RPE cells (Gramajo, 2010). Other oxysterols such as 24-OHC, 25-OHC, 27-OHC, and 7 β -OHC triggered inflammation, induced oxidative stress and apoptotic cell death in various cells (Ares, 2000; Riendeau and Garenc, 2009; Zhang, 1997; Zhou, 1995; Zhou and Kummerow, 1997).

Previous research showed 27-OHC causes AD-like pathology by increasing A β production and triggering apoptotic cell death in human neuroblastoma SH-SY5Y cells (Prasanthi, 2009; Rantham Prabhakara, 2008) and in organotypic slices from rabbit hippocampus (Ghribi, 2009; Sharma, 2008). As 27-OHC shown to cause AD-like pathology by increasing A β production and triggering

apoptotic cell death in human neuroblastoma SH-SY5Y cells and in organotypic slices from rabbit hippocampus, this dissertation work focused on 27-OHC impact on pathological hallmarks that are common to both AMD and AD by using human RPE cell line, ARPE-19.

Mounting evidence demonstrates that oxysterols have deleterious effects that may contribute to the pathogenesis of AD and AMD. However, the intracellular mechanisms underlying oxysterols toxicity are not well known. A common pathological feature that accumulates both in AD and AMD is A β (Dentchev, 2003; Johnson, 2002), a peptide that is considered to play a central role in the neurodegenerative processes by increasing oxidative stress and cell damage. The present study demonstrated for the first time that 27-OHC increases the levels of A β in RPE cells. A β deposition could be an important element in the local inflammatory and oxidative processes that contribute to the deterioration of photoreceptors and pathogenesis of AMD. The APP protein is present in the RPE cytoplasm and A β -labeled structures were also identified in RPE cells (Johnson, 2002), suggesting that RPE cells have the ability to generate A β . A β accumulation observed in this study at least in part is due to increased generation of A β peptide by the action of BACE-1, the enzyme that initiates the cleavage of APP.

Accumulation of A β and oxidative damage are intrinsically related. Oxidative damage in cells can be caused by a variety of factors including endoplasmic reticulum stress, ROS generation, GSH depletion and inflammation. Endoplasmic reticulum stress has been implicated in both AD (Ghribi, 2006) and AMD (Sauer, 2008). The endoplasmic reticulum is the cell compartment where various proteins are synthesized and Ca²⁺ is stored. Cellular stress leads to

depletion of endoplasmic reticulum Ca^{2+} stores, activation of specific endoplasmic reticulum stress proteins such as caspase-12, GADD153 and apoptotic cell death (Oyadomari, 2002). 27-OHC-induced endoplasmic reticulum stress may be due to the loss of Ca^{2+} influx and a decrease in endoplasmic reticulum Ca^{2+} levels in these RPE cells. This was consistent since 27-OHC showed activation of the endoplasmic reticulum specific apoptotic proteins caspase-12 and GADD153. GADD153 is present in the cytosol under normal conditions and translocate to the nucleus following sustained endoplasmic reticulum stress (Ron and Habener, 1992). Induction of GADD153 was shown to upset the cellular redox state by depleting cellular GSH and exaggerating the production of ROS (McCullough, 2001). 27-OHC-induced GADD153 activation is accompanied by increased levels of ROS and depleted glutathione.

HO-1 is mainly located in the ER and stimulates the oxidation of cholesterol to oxysterols. It is regarded as a sensitive marker of oxidative stress in cells and tissues (Hascalovici, 2009; Vaya, 2007). Its induction is suggested to be an early event in the pathogenesis of sporadic AD (Schipper, 2006). HO-1 levels were also increased in RPE of AMD-affected maculas (Frank, 1998). HO-1 gene expression is stimulated by endoplasmic reticulum stress (Liu, 2005). Endoplasmic reticulum stress can also cause apoptosis via NF- κ B activation (Pahl and Baeuerle, 1996). Activation of NF- κ B was observed in AD (Kaltschmidt, 1997) as well as in AMD (Kaarniranta and Salminen, 2009). 27-OHC increased levels of HO-1 and activated NF- κ B. This suggests the potential role of 27-OHC as a pro-oxidant that can cause oxidative damage to retinal cells and in the pathophysiology of AMD.

Increased levels of A β , endoplasmic reticulum stress, HO-1 and NF- κ B are accompanied by glutathione depletion as well as elevated levels of ROS. These results demonstrate the potential oxidative effects of 27-OHC. Increased levels of TNF- α suggest that 27-OHC-induced oxidative stress is associated with triggering of inflammatory processes. All together, oxidative stress and inflammation may ultimately lead to cell death. This study further demonstrates that 27-OHC is cytotoxic, induces the loss of mitochondrial membrane potential and causes apoptotic cell death.

Freshly isolated human retinal pigment epithelial cells and ARPE-19 cells also shown to express ER α and ER β (Giddabasappa, 2010; Marin-Castano, 2003). In one recent study, ER α gene expression was observed in freshly isolated RPE cells but not in ARPE-19 cell culture and primary RPE culture (Dwyer, 2011). In another study, ER α mRNA but not ER β mRNA, was detected in ARPE-19 cells (Paimela, 2010). As cell line establishment and different growth conditions may be responsible for these variances in ER expression, ERs existence was probed in the ARPE-19 cell line used for this work.

In this study, the existence of ER α , ER β , LXR α and LXR β in both cytoplasmic and nuclear fractions of ARPE-19 cells was confirmed by Western blotting. Transfection with ERE-luciferase constructs and LXRE-luciferase constructs and dual luciferase assays upon treatments with ER agonist E2 and LXR agonist GW3965 confirmed ER and LXR transcriptional activity.

CYP27A1 catalyzes the conversion of cholesterol to 27-OHC. A strong stoichiometric correlation has been recognized between circulating levels of 27-OHC and total cholesterol in humans (Brown and Jessup, 1999; Karuna, 2011).

25-OHC is produced from cholesterol primarily by the action of cholesterol 25-hydroxylase. The ability to produce 25-OHC from cholesterol was also reported for sterol 27-hydroxylase and cholesterol 24-hydroxylase (Lund, 1993; Lund, 1999). 25-OHC has also long been recognized as a cholesterol autoxidation product. 7-KC is commonly found in oxLDL associated with atherosclerotic plaques. The light is involved in mediating the oxidation of cholesterol to 7-KC in retina (Rodriguez and Larrayoz, 2010)

Whether oxysterols, 27-OHC, 25-OHC and 7-KC have any impact on ER and LXR mediated transcriptional activity were explored. 27-OHC, 25-OHC and 7-KC inhibited the ERE-luciferase activity. 27-OHC, 25-OHC activated LXRE-luciferase activity, whereas 7-KC did not alter LXRE-luciferase activity. Numerous studies suggested that cytotoxic effects of oxysterols are associated with human diseases including AMD (Vejux, 2008). Increased oxysterol concentrations subsequently to increased cholesterol levels may contribute to the generation of AMD-like pathological hallmarks. 27-OHC, 25-OHC and 7-KC were shown to be cytotoxic to RPE cells by us and others (Dasari, 2010; Luthra, 2006; Ong, 2003). Since these oxysterols also function as ligands of ER and LXR, any potential protective or deteriorating effects of ER and LXR ligands in combination with 27-OHC, 25-OHC and 7-KC were studied.

Epidemiologic studies suggest that the risk for AMD is increased in women undergoing menopause and that women on hormone-replacement therapy have a reduced risk of having the disease (Feskanich, 2008; Friedman, 2004). A relationship between use of postmenopausal exogenous E2 and a lower risk of AMD also suggest a role for estrogen in the pathogenesis of the disease (Marin-

Castano, 2003). ERs were shown to be expressed in the male and female retina (Munaut, 2001; Ogueta, 1999). Both ER subtypes are expressed in human RPE cells and regulated in a dose-dependent fashion by E2 (Marin-Castano, 2003). E2 has been suggested as a potential therapeutic agent in the management of several neurodegenerative disorders because of its actions as an anti-oxidant, anti-apoptotic and anti-inflammatory agent (Dimayuga, 2005; Moosmann and Behl, 1999). E2 treatment was reported to attenuate release of cytokines, reactive oxygen species, and complement proteins (Bruce-Keller, 2000; Drew and Chavis, 2000). These protective effects of E2 prompted us to investigate whether E2 is protective against the oxysterol induced cytotoxicity in ARPE-19 cells.

Our study demonstrate that 27-OHC, 25-OHC and 7-KC were cytotoxic to ARPE-19 cells and treatment with E2 protected against 25-OHC and 27-OHC induced cytotoxicity, whereas ER antagonist ICI 182780 treatment did not offer any protection. However 7-KC induced cytotoxicity was not affected by either E2 or ICI 182780. Then, mitochondrial membrane potential as a marker of mitochondrial stress was examined. 25-OHC, 27-OHC and 7-KC caused mitochondrial membrane potential decline, which is usually observed during apoptosis. E2 treatment counteracted against mitochondrial potential decline in cells incubated with 27-OHC and 25-OHC, but not in cells incubated with 7-KC.

Estrogens inhibit cellular apoptosis through several mechanisms, including acting as nonspecific antioxidants, modulation of gene expression (Strehlow, 2003) and activation of nongenomic signaling pathways (Fernando and Wimalasena, 2004; Urata, 2006; Vasconsuelo, 2008). These mechanisms are dependent on the cell type and pathophysiological situation of the cell.

Several *in vitro* studies have shown that E2 prevents oxidative stress–induced apoptosis of retinal neurons (Simpkins, 2005), RPE cells (Giddabasappa, 2010) and human lens epithelial cells (HLECs) (Flynn, 2008). Apoptosis induction upon oxysterols, 27-OHC, 25-OHC and 7-KC treatment was examined by TUNEL method. 27-OHC, 25-OHC and 7-KC all caused apoptosis. E2 protected against apoptosis in 27-OHC and 25-OHC treated cells, whereas E2 did not offer any protection in 7-KC treated cells. LXR agonist GW3965, antagonist ECHS has not offered any protection against cytotoxicity, mitochondrial membrane potential in oxysterols 27-OHC, 25-OHC and 7-KC treated cells.

Estrogen effects are mostly mediated by ER. Classic ER signaling involves E2 binding to EREs in the promoter regions of target genes. ERs can also modulate non-ERE-containing genes by interacting with the DNA-bound transcription factors. A number of studies have advocated that membrane-bound estrogen receptors cause rapid nongenomic signaling that modify cellular physiology in bone, brain, cardiovascular system, and mammary gland (Levin, 2001). These rapid effects of estrogens are facilitated by the stimulation of signaling cascades such as nitric oxide, receptor tyrosine kinases, G-protein coupled receptors, phosphatidylinositol-3-kinase, serine threonine kinase Akt, mitogen activated protein kinase family members, protein kinase A and protein kinase c (Castoria, 2001; Migliaccio, 1996; Simoncini, 2000). It needs to be determined that the protection of E2 occurs through whether ER α , ER β or non-genomic signaling pathways. Under normal conditions and when oxysterol levels are low, ER function is preserved, whereas conditions which increase these oxysterol levels, such as hypercholesterolemia or conditions that reduce estrogen

levels, ER function could be inhibited by oxysterols. This ER function compromise could explain AMD in conditions related to low levels of E2. These results emphasize importance of potential estrogen receptor agonists for counteracting oxysterol induced toxicity, which could lead to AMD.

All together, this study showed retinal modifications that are relevant to AMD in cholesterol-fed rabbits, a model for AD. Increased levels of A β , decreased levels of the anti-apoptotic protein Bcl-2, increased levels of the pro-apoptotic Bax and GADD153 proteins, emergence of apoptotic cells, and increased generation of reactive oxygen species were found in retinas from cholesterol-fed compared to normal chow-fed rabbits. Additionally, astrogliosis, drusen-like debris and cholesterol accumulations in retinas from cholesterol-fed rabbits were observed. Oxysterol levels in retinas from cholesterol-fed rabbits were increased.

27-OHC dose-dependently increased A β peptide production, increased levels of endoplasmic reticulum stress specific markers caspase 12 and GADD153, reduced mitochondrial membrane potential, triggered Ca²⁺ dyshomeostasis, and increased levels of the NF- κ B and HO-1, two proteins activated by oxidative stress. Additionally, 27-OHC caused glutathione depletion, ROS generation, inflammation and apoptotic-mediated cell death in ARPE-19 cells, a model for retinal pigment epithelial cells. ARPE-19 cells express ER α , ER β , LXR α and LXR β . ERE-luciferase activity is increased by ER agonist E2, and LXRE-luciferase activity is increased by LXR agonist GW3965 confirming ER and LXR transcriptional activity in ARPE-19 cells. ER agonist E2 protected cells from 25-OHC and 27-OHC induced cytotoxicity. E2 counteracted 27-OHC and 25-OHC

induced mitochondrial membrane potential decline. E2 treatment protected cells from 27-OHC and 25-OHC induced apoptosis.

Taken together, data demonstrates that cholesterol-enriched diets induce pathological hallmarks suggestive of AMD in rabbit retinas. The cholesterol diet substantially increased concentrations of the major oxysterols, accumulation of which is toxic to retinal cells. Incubation of cells with oxysterols also reproduced the major effects observed *in vivo* with the diet rich in cholesterol. These results strongly suggest that hypercholesterolemia and subsequent increase in oxysterol formation may contribute to the pathogenesis of AMD. The data also shows that ER modulation may play an important role in the cholesterol and oxysterol effects. Specifically, ER agonists may provide protection against oxysterol deleterious effects on retinas. As well, reduction of cholesterol plasma levels may prevent excess conversion of cholesterol to oxysterols and precludes the incidence of AMD

APPENDIX

22-OHC	22-hydroxycholesterol
24-OHC	24-hydroxycholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
4 β -OHC	4 β -hydroxycholesterol
7-KC	7-ketocholesterol
7 α -OHC	7 α -hydroxycholesterol
7 β -OHC	7 β -hydroxycholesterol
ABCA-1	ATP-binding cassette sub-family A member 1
AC	Amacrine cells
AD	Alzheimer's disease
AMD	Age related macular degeneration
ANOVA	Analysis of variance
APP	Amyloid precursor protein
APS	Ammonium persulfate
ARM	Age related maculopathy
ARPE-19	Arising retinal pigment epithelia
A β	Amyloid beta peptide
BACE-1	Beta-secretase 1
Bax	Bcl-2-associated X protein

BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BM	Bruch's membrane
BP	Bipolar cells
BSA	Bovine serum albumin
CCD	Charge-coupled device
CNV	Choroidal neovascularization
CRALBP	Cellular retinal-binding protein
CRP	C-reactive protein
CYP11A1	Cholesterol side-chain cleavage enzyme, mitochondrial
CYP27A1	Sterol 26-hydroxylase, mitochondrial
CYP3A4	Cytochrome P450 family 3 subfamily A polypeptide 4
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7- α -monooxygenase
DAPI	Diamidino-2-phenylindole
DCFH-DA	Dichlorofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DR	Diabetic retinopathy
E2	Estrogen
EC	Esterified cholesterol
ECHS	5 α -6 α -epoxycholesterol-3-sulfate
ELISA	Enzyme linked immunosorbent assay

ER	Endoplasmic reticulum
ERE	Estrogen response element
ER α	Estrogen receptor α
ER β	Estrogen receptor β
FBS	Fetal bovine serum
GADD153	Growth arrest and DNA damage inducible gene 153
GC	Ganglion cells
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GFAP	Glial Fibrillary Acidic Protein
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GW3965	3-[3-[N-(2-Chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride
H&E	Hematoxylin and eosin
H ₂ O ₂	Hydrogen peroxide
HC	Horizontal cells
HDL	High-density lipoprotein
HO-1	Heme-oxygenase 1
HRP	Horse radish peroxidase
ICI182780	7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol
IDE	Insulin-degrading enzyme

INL	Inner nuclear layer
IPL	Inner plexiform layer
I κ B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JC-1	5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
LDH	Lactate dehydrogenase
LDL	Low-density Lipoprotein
LXRE	Liver X receptor response element
LXR α	Liver X receptor alpha
LXR β	Liver X receptor beta
M-PER	Mammalian protein extraction reagent
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS	Nitric oxide synthase
NP-40	Tergitol-type nonyl phenoxy polyethoxy ethanol
NSAIDs	Nonsteroidal anti-inflammatory drugs
OD	Optical density
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segments
oxLDL	Oxidized LDL
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PR	Photoreceptors

POS	Photoreceptor outer segments
PRL	Photoreceptor layer
PVDF	Polyvinylidene difluoride
RGC	Retinal ganglion cells
ROS	Reactive oxygen species
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate
TEMED	Tetramethylethylenediamine
Tg	Thapsigargin
TNF- α	Tumor necrosis factor- α
T-PER	Tissue protein extraction reagent
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
UC	Unesterified cholesterol
VEGF	Vascular endothelial growth factor

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