ANTIOXIDATIVE ROLE OF AUTOPHAGY IN THE PROTECTION AGAINST *IN VITRO* AND *IN VIVO* OXIDATIVE STRESS-INDUCED MODELS OF AGE-RELATED RETINAL DEGENERATION

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Antioxidative Role of Autophagy in the Protection Against *In Vitro* and *In Vivo* Oxidative Stress-Induced Models of Age-Related Retinal Degeneration

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CERTIFICATE OF ORIGINALITY

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_____ (Signed)

ABOKYI Samuel (Name of Student)

DEDICATION

To my family & friends for your encouragement

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ABSTRACT

Age-related macular degeneration (AMD) is a common cause of visual impairment in the elderly population. There are very limited therapeutic options for AMD with the predominant therapies targeting VEGF-A in the retina of patients afflicted with the "wet form" of AMD. Recently, it is becoming clear that oxidative damage of the retinal pigment epithelium (RPE) is an earlier event in the pathophysiology of AMD. This thesis, therefore, sought to investigate potential molecular mechanisms and targets for protection against oxidative damage in the RPE and retinal degeneration.

With the aim of finding effective cellular defense mechanisms that modulate oxidative stress in RPE cells, this study investigated changes in autophagy and Nuclear factor erythroid 2-related factor 2 (NRF2) in the presence of the hydroquinone (HQ, an oxidant found in cigarette smoke). In vitro, our results showed that human RPE cells were more susceptible to oxidative damage from HQ compared to H_2O_2. The dysregulation of cellular antioxidative pathways such as autophagy and NRF2 were found to be involved. The autophagosome marker LC3-II was increased by both HQ and H_2O_2, suggesting autophagy activation by the oxidants. However, HQ exposure induced NRF2 and p62 inhibition at both transcription and protein levels, unlike H_2O_2. Based on these results, we hypothesized that autophagy upregulation could be an effective approach for inhibiting oxidative damage in human RPE cells.

Next, we assessed the role of autophagy in antioxidants' protection of human RPE cells against oxidative stress. Antioxidant vitamin supplements and the thiols are recommended for managing AMD. We demonstrated in this study that vitamin supplements and thiols upregulated autophagy flux via the transcription factor EB (TFEB), resulting in their protection against HQ- or H_2O_2 -induced oxidative damage in human RPE cells.

Trehalose is a natural dietary molecule reported to induce autophagy and antiaging and neuroprotective effects in several animal models of neurodegenerative diseases. Hence, we investigated the protective effect of trehalose-induced autophagy against oxidative stress in human RPE cells. Incubation of the cells with trehalose upregulated autophagy-related genes and protein markers of macroautophagy and chaperone-mediated autophagy (CMA). Additionally, we found that trehalose rescued human RPE cells from HQ-induced oxidative damage in an autophagy dependent manner. These findings warranted investigation into the neuroprotection of trehalose in oxidative stress-mediated retinal degenerative diseases.

Lastly, we studied the contribution of autophagy to oxidative stress and the development of retinal degeneration in normal ageing wild-type BALB/c mice. Mice monitored from age 2-18 months, using electroretinography (ERG) and spectral-domain optical coherence tomography (SD-OCT), demonstrated retinal degenerative changes in older mice which were accompanied with autophagy decline and increased oxidative stress. Immunoblot and quantitative real-time PCR results showed a downregulation of autophagy-related genes and/or proteins including ATG5 and ATG7, LC3-II, LAMP-2A, and TFEB in the neural retina of older mice with degenerative changes. Oral administration of trehalose improved TFEB-mediated autophagy and cone-mediated response decline associated with aging in BALB/c mice.

Collectively, our data reveal the significance of TFEB-mediated autophagy in the protection of human RPE cells and retina against oxidative damage. More importantly, it supports the targeting of TFEB-induced autophagy as a novel therapeutic approach for preventing age-related decline in cone-mediated function.

LIST OF PUBLICATIONS

The thesis contains a list of manuscripts that may be accepted for publication by a journal, under review by a journal or still being prepared.

Paper I: Central role of oxidative stress in age-related macular degeneration: Evidence from review of the molecular mechanisms and animal models. (Published) *Oxidative Medicine and Cellular Longevity journal.* Pages 13-51.

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Paper III: Vitamin C and N-acetyl cysteine induced TFEB-dependent autophagy and NRF2 for protection against oxidative stress: implications for antioxidant supplements in neurodegeneration. Manuscript under preparation. Pages 81-106.

Paper IV: Autophagy upregulation by the TFEB-inducer trehalose protects against oxidative damage and cell death associated with NRF2 inhibition in human RPE cells. (Published) *Oxidative Medicine and Cellular Longevity journal.* Pages 108-136.

Paper V: Trehalose improves TFEB-mediated autophagy and cone photoreceptor function decline associated with aging in BALB/c mice. Manuscript under preparation. Pages 138-163.

Paper VI: Emerging role of autophagy in age-related macular degeneration: a review of literature. Manuscript under preparation. Pages 2-8.

CONFERENCE PAPERS (PUBLISHED)

1. Abokyi S, To C, Shan SSW, Chan HH, Tse D

Cigarette smoke oxidant, hydroquinone, downregulates p62 and NRF2 protein expressions in human RPE and induces cell death.

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2. Abokyi S, To C, Shan SW, Chan HH, Tse DY

Molecular mechanisms associated with the protective effect of the disaccharide trehalose against oxidative damage in the human retinal pigment epithelial cells.

Investigative Ophthalmology & Visual Science 60 (9), 1949-1949.

Presented at the Association for Research in Vision and Ophthalmology (ARVO) 2019 Annual Meeting, April, 28 - May 2, in Vancouver, Canada.

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LIST OF SPECIFIC PRIMERS

Table 1: List of primers used for investigating gene expression levels

Gene	*Primer Sequence*
Human β-actin	Forward primer: 5′-CCAACCGCGAGAAGATGA-3′ Reverse primer: 5′-CCAGAGGCGTACAGGGATAG-3′
Human TFEB	Forward primer: 5’-CCAGAAGCGAGAGCTCACAGAT-3’ Reverse primer: 5’-TGTGATTGTCTTTCTTCTGCCG-3’
Human ATG5	Forward primer: 5’-AAGCTGTTTCGTCCTGTGGC-3’ Reverse primer: 5’-CCGGGTAGCTCAGATGTTCA-3’)
Human ATG7	Forward primer: 5’-CGTTGCCCACAGCATCATCTTC-3’ Reverse primer 5’-TCCCATGCCTCCTTTCTGGTTC-3’
Human NRF2	Forward primer: 5′-ACACGGTCCACAGCTCATC-3′ Reverse primer: 5′-TGTCAATCAAATCCATGTCCTG-3′
Human p62	Forward primer: 5′-TGCCCAGACTACGACTTGTG-3′ Reverse primer: 5′-AGTGTCCGTGTTTCACCTTCC-3′)
Human VEGF-A	Forward primer: 5’-TGCCATCCAATCGAGACCCTG-3’ Reverse primer: 5’-GGTGATGTTGGACTCCTCAGTG-3’
Human Hsp27	Forward primer: 5′-TCCCTGGATGTCAACCACTT-3′ Reverse primer: 5′-GATGTAGCCATGCTCGTCCT-3′

ABBREVIATIONS

A2E	N-retinylidene–N- retinyl ethanolamine
AMD	Age-related macular degeneration
AMPK	5' adenosine monophosphate-activated protein kinase
ATG	Autophagy-related gene
ATP	Adenosine triphosphate
CEP	Carboxyethylpyrrole
CLEAR	Coordinated Lysosomal Expression and Regulation
CMA	Chaperone-mediated autophagy
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CQ	Chloroquine
CSE	Cigarette smoke extract
DHA	Docosahexaenoic acid
ERG	Electroretinogram/electroretinography
GCL	Ganglion cell layer
GSH	Glutathione
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase 1
HQ	Hydroquinone
Hsc70	Heat shock cognate 70 kd protein
ICAM	Intercellular adhesion molecule
IL	Interleukins
IL	Interleukin
INL	Inner nuclear layer
IPL	Inner plexiform layer
KRT*8*	*Keratin-8 protein*
LAMP2	Lysosomal membrane protein 2
LC3	Microtubule-associated proteins 1A/1B light chain 3B

LCN-2	Lipocalin-*2*
MCP-1	Macrophage cationic peptide
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of Rapamycin
NAC	N-acetyl cysteine
NFL	Nerve fiber layer
NH_4Cl	Ammonium chloride
NLRP3 inflammasome	NACHT, LRR and PYD domains-containing protein 3
NRF2/ NF2EL2	Nuclear factor erythroid 2-related factor 2
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PFA	Polyunsaturated fatty acid content
PL	Photoreceptor layer
POS	Photoreceptor outer segment
RGC	Retinal ganglion cells
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SDF-1	Stromal cell-derived factor-1
SD-OCT	Spectral-domain optical coherence tomography
shRNA	Short hairpin RNA
SOD	Superoxide dismutase
TFE3	Transcription factor binding to ighm enhancer 3
TFEB	Transcription factor EB
TRE	Trehalose
VEGF	Vascular endothelial growth factor
VIT C	Vitamin C
VIT E	Vitamin E
8-OHdG	8-hydroxy-2-deoxyguanosine

CHAPTER I

Introduction

Background

AMD is a neurodegenerative disease that affects the central retina in the aging eye, resulting in progressive loss of vision. It is among the commonest causes of visual impairment and disability in the aging population (Chou et al., 2013). Clinical presentation of early AMD is similar in every person. In the eye, fundoscopy reveals the accumulation of sub-retinal pigment epithelial (sub-RPE) deposits including drusen and basal membrane deposits (Bhuiyan et al. 2013; Khan et al. 2016). These clinically observed retinal changes are associated with damage to the photoreceptors and retinal pigment epithelium (RPE). In AMD, the death of photoreceptors is by apoptosis (Vihtelic & Hyde, 2000; Wenzel et al., 2005). At the early stage vision may be minimally impaired but in most people is unnoticed. However, studies investigating visual function in early AMD have reported a decline in high-contrast visual acuity, colour vision, contrast sensitivity, and rod-mediated multifocal electroretinograms compared to their age-matched normal cohorts (Feigl et al. 2005a; Feigl et al. 2005b). Also, Midena and co-workers have shown that a delay in macular recovery function occurs in early AMD (Midena et al. 1997). There is, however, a remarkable difference among persons with the advancement of AMD; the disease mostly progresses into a form referred to as dry AMD but in others, wet AMD develops. Dry AMD is characterized by geographic atrophy seen by fundoscopy as a sharply demarcated area of depigmentation due to loss of the RPE. Choroidal neovascularization is the main feature in wet AMD, noticed as an area of subretinal hemorrhage and intrusion of blood vessels at the central retina (Bird et al. 1995). Both advanced stages of AMD are always

associated with severe vision loss, which is more rapidly progressive in the wet form (Coleman et al. 2008).

Management of age-related macular degeneration

Since AMD is a progressive condition, its treatment could focus on either preventing further damage to the retina and/or restoring damaged retinal tissue. The retina, like other nervous tissue, lacks the ability to self-regenerate after damage. Clinical trials have shown promising results on the potential to regenerate the RPE from embryonic stem cells, induced pluripotent stem cells, and undifferentiated retinal progenitors from the bone marrow or umbilical cord tissue (Kanemura et al., 2014; Schwartz et al., 2015). Studies suggest sheets of RPE can be grown and implanted into the retina to replace damaged RPE and improve vision in retinal disorders including age-related macular degeneration, Stargardt's disease and retinitis pigmentosa in the short to medium term (Schwartz et al., 2015). However, there could be complications related to the possibility of mycoplasma contamination, purity of the differentiated RPE colonies, tumorigenicity due to plasticity and unlimited capacity for self-renewal, and immune rejection, which could be sight and life-threatening (Nazari et al., 2015).

Besides the regenerative medicine approach that could be beneficial for all forms of AMD, an alternative treatment option in dry AMD is the Age-Related Eye Disease Study (AREDS) formulation (Chew et al., 2012; Kassoff et al., 2001). The supplement formula comprises vitamins C and E as the mainstay treatment, plus Zinc or carotenoids which are optional. According to the available data, this treatment modality could delay the risk of vision loss in some people with intermediate to advanced dry AMD (Chew et al., 2012; Kassoff et al., 2001). It is, however, important to note that the results from different studies about these benefits of the formula in AMD management are conflicting and need further investigations (Jennifer R. Evans & Lawrenson, 2017).

For wet AMD, treatment modalities include laser photocoagulation and anti-angiogenic drugs that may be prescribed singly or as a combination therapy. Laser photocoagulation uses high-energy laser light to destroy actively growing abnormal new blood vessels in retina. The anti-angiogenic medications which are mainly anti-VEGF agents, such as pegaptanib, ranibizumab, bevacizumab and aflibercept, are injected monthly or bimonthly into eyes to stop the growth of new blood vessels and to block blood leakage that worsen vision loss in wet AMD (Al-Zamil & Yassin, 2017). Notwithstanding the efficacy, the laser treatment has limitations which include the recurrence of abnormal blood vessel growth within 2 years as well as irreversible RPE and retina damages, leading to an absolute scotoma at the site of the laser photocoagulation scar (Ciulla et al., 1998).

Emerging role of autophagy in age-related macular degeneration

Our current understanding of autophagy can be traced back to the work of Nobel Laureate Christian de Duve (1974) in 1955, who discovered lysosomes as an intracellular digestive organelle in eukaryotic cells and coined the term autophagy in 1963 (Baiton, 1981). Subsequently, it was discovered that lysosomes contained diverse acid hydrolases, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases, and lipases, that facilitate the degradation of almost all classes of macromolecules including proteins, lipids, and genetic materials (DNA and RNA), and the recycling of the nutrients back to the cells (Mony et al., 2016; Schröder et al., 2010). Significant insight into autophagy was gained following the characterization of the essential autophagy-related genes (ATG) in yeast in the 1990s by Yoshinori Ohsumi (Levine & Klionsky, 2017), who was also awarded a Nobel prize in 2016. With the work of many investigators, we started to understand the role of individual Atg proteins

and their contributions to each of the stages of the autophagy process, starting from initiation to autophagosome maturation, and finally to autolysosome formation (Hurley & Young, 2017; L. Yu et al., 2018).

There are three types of autophagic processes depending on the route through which substrates are delivered to the lysosomes for degradation, namely microautophagy, chaperone-mediated autophagy, and macroautophagy (Fig. 1.1). Microautophagy is the simplest kind that involves direct uptake of cytoplasmic material into the lysosome by means of invagination or cellular protrusion of the lysosomal membrane (W. W. Li et al., 2012). Chaperone-mediated autophagy is more selective and complex compared to microautophagy due to the involvement of the lysosome-associated membrane protein 2 (LAMP2) and the Hsc70 protein complex, which binds to only KFERQ-motif bearing substrates in the cytosol for transport across the lysosome-associated membrane into the lysosome for processing (Susmita Kaushik et al., 2011). Macroautophagy is the most complex of the three processes. There are the recognition and binding of a cytosolic substrate by the autophagy adaptor p62/SQSTM1 for sequestration within autophagosomes, a double membrane-bound vesicle. Next, the matured autophagosomes fuse with the lysosome for degradation (L. Yu et al., 2018). This catabolic process, known as macroautophagy but simply referred to as autophagy in this text, is considered essential for the recycling of important molecules from damaged or long-lived molecules and organelles contributing to cell homeostasis and survival. This recycling role becomes highly significant especially in conditions of excessive stress as in periods of starvation or stress such as growth factor deprivation. For instance, during nutrient starvation in yeasts autophagy is upregulated, resulting in degradation of unneeded proteins into amino acids, which are recycled for the synthesis

of proteins that are essential for survival (Farré & Subramani, 2016; Zimmermann et al., 2016).

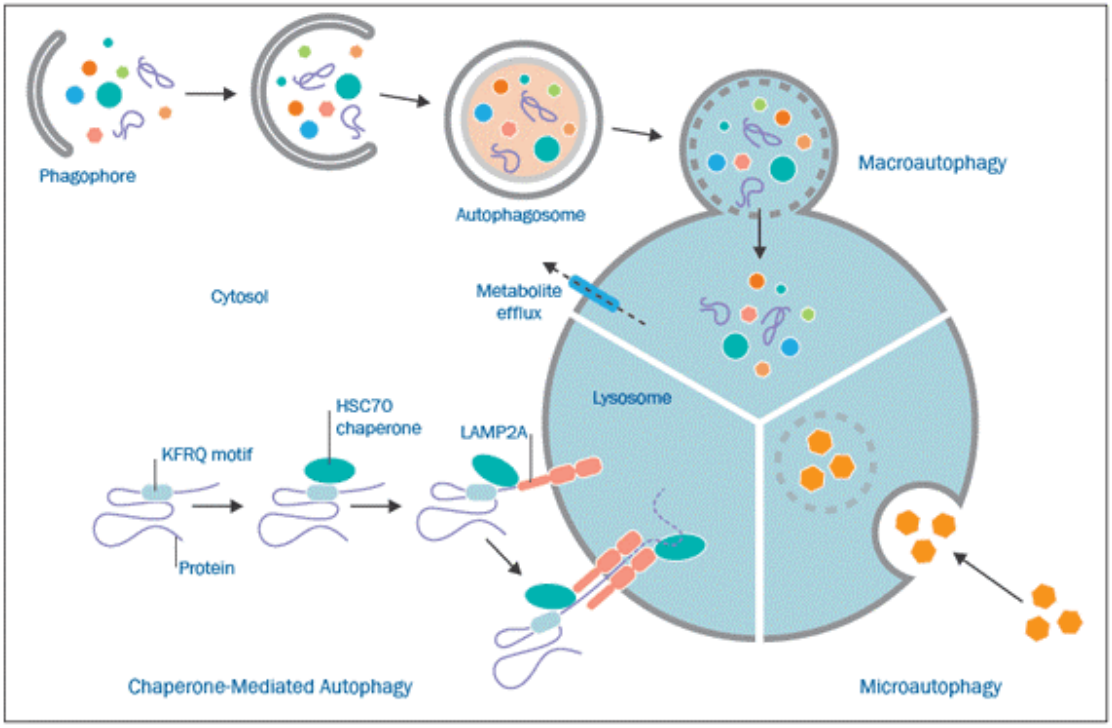


Figure 1. 1: The 3 different pathways involved in the lysosomal degradation of intracellular substrates (referred to as autophagy).

Evidence of dysregulated autophagy in AMD

Findings from studies using donor specimens from AMD subjects and experimental models of the disease support the involvement of autophagy. Lipofuscin and drusen deposits, composed of poorly degraded protein and lipid substrates in the RPE, are the most significant risk factor in the development of AMD (Crabb, 2014; Sakurada et al., 2018). The increased presence of these deposits in the AMD retinas is a strong indication of an inefficient autophagic degradation since the autophagy pathway is responsible for the clearance of aggregated mutant proteins in disease models characterized by proteinopathies (Metcalf et al., 2012; Sarraf et al., 2019; Thellung et

al., 2018). In vitro studies on human RPE cells demonstrated that intimate relationship between autophagy and lipofuscin levels, by showing that lipofuscin level was increased following the inhibition of autophagy, and higher lipofuscin levels also inhibit autophagy (Höhn & Grune, 2013; Lei et al., 2017; Mitter et al., 2014).

Apart from the aggregation of retinal deposits, imputing the dysregulation of autophagy in AMD, other studies have also found differential expressions of specific autophagy makers in cultured primary RPE cells and in drusen deposits from postmortem eyes with AMD compared to healthy non-AMD eyes (Golestaneh et al., 2017; Mitter et al., 2014; Ai Ling Wang et al., 2009). The autophagy protein, ATG5, was found to be highly expressed in drusen from the retina of AMD donors compared to that of healthy non-AMD cohorts, which was interpreted as upregulated autophagy (A L Wang et al., 2009). An elevated ATG5 may, however, not necessarily translate into an increase in the autophagy flux (Klionsky et al., 2012), On the contrary, Golestaneh et al. reported reduced levels of the autophagosome marker LC3-II and increased levels of the autophagy substrate p62 and lysosome-associated membrane protein-1 (LAMP-1) during starvation in cultured RPE cells from AMD eyes than that of normal non-AMD eyes (Golestaneh et al., 2017), pointing to impaired autophagy in AMD (Klionsky et al., 2008, 2012). Mitter and coworkers, however, showed that at the early stage of AMD autophagy was upregulated in the retina and become down-regulated in advanced stages AMD of the disease (Mitter et al., 2014). Collectively, this evidence supports the involvement of autophagy in AMD, and continuous investigation may be helpful to understand why there is downregulation in the autophagy activity level in the AMD retina when upregulating autophagy could have reduced the burden of oxidized biomolecules and mitochondria (Golestaneh et al., 2017; Liguori et al., 2018).

Role of autophagy in models of AMD

The specific role of autophagy in AMD pathophysiology is yet to be fully understood, and most of what is currently known come from studies conducted using animal models and cell cultures. However, evidence continues to mount in support of autophagy as an essential mechanism promoting cell survival under conditions of stress or diseases including AMD (Giansanti et al., 2013; Mitter et al., 2014; J. Viiri et al., 2013). In studying the role of autophagy, studies employed either pharmacological or genetic modulators to induce or inhibit autophagy. Some recent studies used the genetic manipulations of ATG as a modulator. The genetic approach might be preferred as it eliminates potential confounders likely to arise from the unwanted side effects associated with pharmacological agents.

Retinoblastoma coiled-coil protein 1 (RB1CC1), Atg5 and Atg7 play crucial roles in autophagy induction in humans (Morselli et al., 2011; Nishimura et al., 2013). Atg5 and Atg7 are involved mainly in the early membrane nucleation stage whereas RB1CC1 is noted to regulate both the early and the late events of autophagosome formation (S. Li et al., 2016; Shimizu et al., 2010). Yao et al. (2015) showed that in the knockout of RB1CC1 in the RPE of mice caused phenotypic signs of AMD including the accumulation of oxidized proteins and lipids, lipofuscin, and complement component C3. In addition, there was a loss of RPE and photoreceptors, retinal microglial cell infiltration, and sub-RPE deposits in the knock-out mice's retinas. Likewise, Zhang et al. (2017) reported that >8 months old mice with deleted RPE-specific Atg5 and Atg7 showed retinal damage including abnormal RPE thickness and photoreceptor degeneration, but without sub-RPE deposits (Y. Zhang et al., 2017). These rodent models of retinal degenerations elucidate the essential role of the basal level of

autophagy in keeping intracellular homeostasis in normal cells and the maintenance of a normal healthy retina. Also, Liu et al. found that intravitreal injection of mice with wortmannin, an autophagy inhibitor, resulted in the death of RPE and photoreceptor cells, followed by subretinal recruitment of inflammasome-activated macrophages, the CCR2 macrophages which release chemokines CXCL1 and CCL2 (J. Liu et al., 2016a). They further demonstrated that in vitro that the malfunctioning of the RPE cells, due to dysregulation of autophagy, triggered macrophage inflammasome activation, release inflammatory cytokine under oxidative stress (J. Liu et al., 2016a). Likewise, several in vitro studies support the role of autophagy in regulating oxidative stress and inflammation (Mitter et al. 2014; Piippo et al. 2014). It was shown that incubating cultured RPE cells with the autophagy inhibitor Bafilomycin A1 caused the aggregation of the oxidized hydroxynonenal-protein adduct and an elevation of levels of inflammatory mediators including NLRP3 inflammasome, IL-1β, and IL-18 in the cells (Piippo et al., 2014).

Even though the benefits of autophagy have been demonstrated mainly in living systems where autophagy is inhibited, there is a growing body of evidence from experimental studies also in support of neuroprotection by the upregulation of autophagy (Yu Chen et al., 2013; Wei et al., 2018; L. Xu et al., 2018). An AMPK activator and autophagy inducer, Metformin, was protective photoreceptors and the retinal pigmented epithelium (RPE) in three different mouse models of retinal degeneration, including acute bright light damage, *Pde6b*^{rd10} inherited retinitis pigmentosa, and sodium iodate-induced RPE injury (L. Xu et al., 2018). Also, the drug 17β-estradiol was found to protect against blue LED-induced retinal degeneration in rats by inducing autophagy (Wei et al., 2018). The protective role of autophagy has

been associated with its function as a quality control mechanism in the removal of intracytoplasmic aggregate-prone proteins and damaged mitochondria (Ho Yoon & Chung, 2019; Um & Yun, 2017; Vucicevic et al., 2018), as well as other by regulation of inflammation and angiogenesis through other autophagy-related downstream signaling pathways (Deretic & Levine, 2018; Qian et al., 2017; X.-R. Zhu & Du, 2018).

Conclusion and perspectives

Largely, the role of autophagy in AMD, as well as other neurodegenerative diseases, has been found to be protective. This evidence is based on the data that demonstrates that upregulating autophagy inhibits molecular pathways including oxidative stress, inflammation, and angiogenesis, implicated in the pathogenesis of AMD. Therefore, identification of pharmacological substances capable of inducing autophagy in the retina might be a laudable approach to finding an effective treatment for the management of AMD.

Problem statement

Based on the need for preventive therapy against retinal degeneration, targeting the molecular mechanisms involved in the development of AMD might be the right approach to finding an effective treatment. Numerous molecular mechanisms are reported to contribute to the development of AMD, including oxidative stress, dysregulated angiogenesis, inflammation and dysregulated lipid metabolism (Bressler, 2009; Jarrett & Boulton, 2012; M. Nowak et al., 2005; Telander, 2011). In fact, it is demonstrated in vivo in experimental animals that dysregulation of any of these molecular mechanisms in the retina could lead to the occurrence of some form of retinal degeneration (Pennesi et al., 2013). Of the several mechanisms, however, literature strongly supports that oxidative stress plays a very central role in the development of AMD. Evidence supporting this assertion could be deduced from the finding that serum from AMD subjects show increased level of oxidative stress (Totan et al., 2009). Also, the RPE from AMD donors' eyes show accumulation of oxidatively damaged biomolecules as well as increased production of reactive oxygen species (Golestaneh et al., 2017). In addition, epidemiological studies have established that cigarette smoke, a source of the pro-oxidant hydroquinone, is a major risk factor for AMD (Cho et al., 2014; Myers et al., 2014). Since cross-sectional studies are limited in showing whether oxidative stress is a cause or consequence of retinal damage, other researchers conducted experimental studies to clarify the relationship between oxidative stress and AMD. The evidence demonstrated that oxidative damage was an important pathophysiological mechanism capable of inducing inflammation and choroidal neovascularization in the retina (Y.-W. Kim & Byzova, 2014; Pujol-Lereis et al., 2016; West et al., 2010). Under conditions of oxidative stress, there is an upregulation of VEGF-A and downregulation of CFH in the RPE, responsible for inducing choroidal

neovascularization and inflammation, respectively (Marazita et al., 2016). Experimentally too, it is demonstrated that impairment of important cellular antioxidant mechanisms such as autophagy and NRF2 signaling pathway is capable of inducing age-dependent retinal degeneration (Yao et al., 2015; Z. Zhao et al., 2011).

Autophagy, a cellular degradation process involved in the recycling of obsolete cellular constituents and the elimination of damaged biomolecules, including protein, DNA, and lipids, is found to promote survival in adverse conditions and enhance longevity (Ohsumi, 2014). Due to the current understanding of the importance of this machinery in health and its potential utilization, autophagy was awarded a Noble Prize in medicine in 2016; credited to Yoshinori Ohsumi for his significant contribution to the identification of autophagy genes and their respective roles (Levine & Klionsky, 2017). The role of autophagy in neurodegenerative diseases is evident. Firstly, most of the prevalent neurodegenerative disorders in humans involve protein misfolding and the aggregation of specific proteins, suggesting that autophagy may be compromised or overwhelmed by the demand (Martinez-Vicente, 2015; Menzies et al., 2015). Some of these diseases include Alzheimer's disease (AD), Parkinson's disease (PD), as Huntington disease (HD). Next, although neurodegeneration in humans is usually associated with aging some could be induced by the inhibition of autophagy, an indication that autophagy and aging may be related (Rubinsztein et al., 2011). The association between autophagy and aging is supported by the observation that upregulating autophagy increases lifespan in model organisms while its inhibition compromises the longevity. Lastly, Findings from animal studies demonstrate that the upregulation of autophagy could be effective in the treatment of some neurodegenerative diseases. Considering that human AMD is similar to other neurodegenerative diseases in terms of the increased accumulation of modified proteins

and mitochondria, it is reasonable to speculate that autophagy may be protective against AMD. In fact, few studies have recently demonstrated that autophagy in the RPE is essential for homeostasis of the retina and that its impairment could contribute to retinal degeneration (Yao et al., 2015; J Zhang et al., 2015).

Numerous natural autophagy enhancers have been reported, the few common ones include resveratrol, curcumin, Kaempferol and trehalose (Z.-Y. Wang et al., 2017). Among these, the autophagy-induced neuroprotective effect of trehalose has been investigated in several neurodegenerative diseases. Preclinical data support the amelioration of toxic protein aggregates by trehalose in animal models of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and Huntington's disease (Hosseinpour-Moghaddam et al., 2018; Khalifeh et al., 2019). In addition to the recently found TFEB-dependent autophagy induction, the disaccharide has a protein stabilizing effect through its action as a chaperone which inhibits the aggregation of misfolded proteins and cell death (N. K. Jain & Roy, 2009). Moreover, trehalose has been approved as a safe food for humans by the European regulatory system and the U.S. Food and Drug Administration in 2000 (Richards et al., 2002). This disaccharide has already been used in clinical trials as a topical and systemic treatment in ocular and systemic disoders, including oculopharyngeal muscular dystrophy, spinocerebellar atrophy type 3, dry eyes, and after laser-assisted in situ keratomileusis (Argov et al., 2015; W. Chen et al., 2009; Mateo et al., 2017; Noorasyikin et al., 2020). It was, therefore, hypothesized that trehalose might be neuroprotective against oxidative stress-induced retinal degeneration. This is, therefore, a translational study whose outcome has direct implications in the current management of AMD.

Aim of the study

To study the role of autophagy in cytoprotection and age-related retinal degeneration.

Specific objectives

1. To explore the dysregulation of cellular antioxidant defense mechanisms in oxidative damage induced by hydroquinone in human RPE cells.
2. To determine the effect of recommended AMD antioxidant treatments on autophagy and its role in the cytoprotection against oxidative stress in human RPE cells.
3. To investigate autophagy induction by trehalose and show its cytoprotection against hydroquinone-induced damage in human RPE cells.
4. To examine the contribution of TFEB-induced autophagy decline on oxidative stress and age-related retinal degenerative changes and the neuroprotective effect of trehalose in BALB/c mice.

CHAPTER II

Central role of oxidative stress in AMD: Evidence from review of the molecular mechanisms and animal models

Chapter summary

Age-related macular degeneration (AMD) is a common cause of visual impairment in the elderly. There are very limited therapeutic options for AMD with the predominant therapies targeting VEGF-A in the retina of patients afflicted with wet AMD. This study, therefore, provides a comprehensive review of studies on human specimens as well as rodent models of the disease, to identify and analyze the molecular mechanisms behind AMD development and progression.

I wish to explicitly state that part of this chapter has been published as a review paper and should be appropriately referenced in case of use as "Samuel Abokyi, Chi-Ho To, Tim T Lam, Dennis Y Tse. Central Role of Oxidative Stress in Age-Related Macular Degeneration: Evidence from a Review of the Molecular Mechanisms and Animal Models. Oxid Med Cell Longev. eCollection2020: 7901270."

Introduction

Age-related macular degeneration (AMD) is a neurodegenerative disease that affects the central retina of an aging eye, resulting in progressive loss of vision and a common cause of visual impairment and disability in the aging population (Chou et al., 2013). The global burden of AMD is estimated at 8.7% and dry AMD accounts for approximately 90% of the total number of people with this vision-threatening condition (Wong et al., 2014). Currently, anti- vascular endothelial growth factor (VEGF) therapy is approved only for the treatment of the wet form of AMD and involves the inhibition

of VEGF-A to VEGF receptors in the retina. The search for an effective treatment for dry AMD is still ongoing and depends on the understanding of the sequence of molecular mechanisms that are involved in the pathogenesis of this eye disease.

Studies in human populations and AMD donor eyes from AMD patients have provided significant insight into the understanding of the pathogenesis of AMD. Evidence indicates that AMD is a multifactorial disease, having both genetic and environmental risk factors (Sobrin & Seddon, 2014). The risk of AMD is greater in persons with family history than those without (Shahid et al., 2011). Observational studies have identified major environmental risk factors such as cigarette smoking, obesity, nutritional factors, and alcoholism (Sobrin & Seddon, 2014). However, investigation of the pathogenesis of AMD is limited by the inability to study the molecular mechanisms involved as they might have happened long before the diagnosis of the condition. Also, it is challenging to study this condition because of the complex nature of AMD which may arise from interactions among those risk factors involved. Hence, the use of animal models of retinal degeneration under controlled conditions in studying AMD provides crucial insight into the disease. In addition, the inducement of retinal degeneration in animals takes a relatively shorter time and provides prompt information than studying AMD in humans. As a result, studies on animal models have played a pivotal role in the preclinical evaluations of interventions, such as anti- VEGF treatments in neovascular AMD, before trials of such treatments in human (Meyer & Holz, 2011).

Experimental models of AMD have been established in many species including Drosophila, mice, rats, guinea pigs and monkeys. While the primate models may be preferable due to their similarities in retina structure and drusen formation and composition with humans (Umeda et al., 2005), the longer time required for inducement

and challenge in breeding them make the murine models much preferred for studying AMD because of lower cost, fast disease progression and ease of genetic engineering. However, no existing animal model yet fully recapitulates the retinal changes found in human AMD. Notwithstanding, the rodent (murine) models show retinal changes including subretinal deposits, thickening of the Bruch's membrane (BrM), loss of retinal pigment epithelium (RPE) and photoreceptors, and choroidal neovascularization (CNV), which are characteristic of AMD (A. Bird et al., 1995).

The objective of this review was to evaluate evidence in support of the involvement of oxidative stress, inflammation, dysregulated lipid metabolism, and dysregulated angiogenesis in the pathogenesis of AMD, relying on the information from human studies and existing animal models of AMD, to help illustrate the roles of these mechanisms. The strength and pitfalls of each animal model were reviewed to assist inform in the selection of suitable models for investigating any of the molecular mechanisms. We demonstrated the primary role that oxidative stress may play in triggering each of the mechanisms and illustrated why the targeting of mechanisms including autophagy, NRF2, and lipid metabolism in the retina might be the futuristic research direction to explore in the search of treatment for AMD.

1.1 Overview of AMD pathobiology

The histopathology of the AMD retina reveals that this ocular disease is characterized by localized destruction of the retinal layers at the macular region, including the RPE and photoreceptors, as well as the adjacent BrM and choriocapillaris. Retinal changes observed in the AMD eyes are varied and include 1) loss of the RPE and photoreceptor layer 2) accumulation of lipids and protein deposits beneath the RPE or in the BrM, and 3) choriocapillaris dropout, CNV and disciform scarring (Mcleod et al., 2009; Zarbin,

2004). In addition to these, are inflammation response through the recruitment of macrophages and microglia, and complement activation (Gupta et al., 2003; Natoli et al., 2017).

Although controversial, most studies have proposed the RPE as the primary site of injury in AMD (Mettu et al., 2012; Zarbin, 2004). The RPE *in situ* performs several functions to maintain retinal homeostasis, some of which include: (1) regulating the transport of nutrients and metabolites, (2) absorption of light, (3) recycling of the retinal visual pigment for the continuity of phototransduction and (4) phagocytosis of shed photoreceptor outer segments. Experimental data support that a malfunction of the RPE leads to retinal degeneration in animal models (Strauss, 2005). One of early molecular event believed to be associated with RPE malfunction in AMD is the age-related accumulation of lipofuscin, a remnant from poorly degraded phagocytosed photoreceptor outer segments (J. Z. Nowak, 2006; Sparrow & Boulton, 2005). Lipofuscin in the RPE may contribute to oxidative damage through the generation of free radicals, as well as inhibition of phagocytotic degradation of damaged biomolecules and organelles (Finnemann et al., 2002; Glickman et al., 1999; Höhn & Grune, 2013; Olchawa et al., 2017).

Adjacent to the RPE pathological changes in AMD eyes is also extracellular deposits, which include the basal lamina deposit, basal linear deposits, and drusen. Drusen and other basal deposits in the BrM are important risk factors in the development of AMD (C. A. Curcio & Millican, 1999). Generally, two processes are believed to contribute in the formation of the subretinal lipid/protein deposits: 1) inefficient RPE metabolism, inefficient degradation of substrate, or damaged RPE cells give rise to debris, and 2) local chronic inflammation due to complement activation by activated microglia recruited to the site of debris (Anderson et al., 2002; Lincoln V Johnson et al., 2001;

Natoli et al., 2017; Umeda et al., 2005). Apart from the immunogenic properties, these lipid deposits become easily oxidized contributing to oxidative stress (Booij et al., 2010; Hageman et al., 2001). In addition, the BrM serves as a semipermeable membrane facilitating the diffusion of nutrients and metabolites between the outer retina and the choriocapillaris; hence, accumulation of deposits within the BrM impairs the transportation of molecules and lead to damage of the RPE and photoreceptor layers (Booij et al., 2010; Guymer et al., 1999). While pathological changes in the RPE may be widely accepted to be the earliest damage in AMD, it was argued by some researchers that the pathophysiology in AMD may differ between the dry AMD and wet AMD (A. C. Bird et al., 2014; Mcleod et al., 2009). McLeod and coworkers reported that in dry AMD, regions of complete RPE atrophy in the AMD retina preceded the adjacent areas of loss of in the choriocapillaris, pointing to the RPE as the primary site of insult. On the contrary, they observed that in wet AMD or choroidal neovascularization (CNV), choriocapillaris loss preceded RPE atrophy, implicating the choriocapillaris as the focus of injury in wet AMD, which in turn could induce hypoxia in the adjacent RPE, upregulating vascular endothelial growth factor (VEGF), and promoting CNV (Mcleod et al., 2009).

Molecular mechanisms and models of AMD

Evidence suggests that AMD is a complex disease having multiple risk factors and molecular mechanisms. The studies on experimental models of AMD suggest that these molecular mechanisms involved in AMD could be categorized broadly into (1) oxidative stress-mediated (Beatty et al., 2000; Jarrett & Boulton, 2012), (2) dysregulated antioxidant mechanisms (3) inflammation (Donoso et al., 2006; Kauppinen et al., 2016), (4) dysregulated lipid metabolism (Dithmar et al., 2000;

Ebrahimi & Handa, 2011; Kishan et al., 2011), and (5) dysregulated angiogenesis (Penn et al., 2008; A. Witmer, 2003). This review focuses on recent developments that explain each of these mechanisms in AMD and in particular describes the various murine models employed in these studies.

Oxidative stress and AMD

Oxidative stress appears to be central in the development of AMD due to its relationship with other molecular mechanisms found in AMD. Generally, oxidative stress is characterized by increased levels of reactive oxygen species (ROS) resulting in the damage or modification of cellular proteins, lipids, and DNA, thereby impairing their physiological roles (Kohen & Nyska, 2002). Several physiologic conditions favor the generation of ROS and oxidative stress in the retina, which includes higher oxygen metabolism, higher polyunsaturated fatty acid content (PFA), and presence of photosensitive molecules (rhodopsin and lipofuscin) and retinal illumination (Beatty et al., 2000).

A huge body of literature supports the involvement of oxidative stress in AMD. Blood serum from AMD subjects showed increased levels of oxidative stress indicated by increased levels of malondialdehyde (MDA), protein carbonyls and 8-hydroxy-2-deoxyguanosine (8-OHdG) compared to normal non-AMD cohorts (Totan et al., 2009). Thus, suggesting that systemic oxidative stress is related to AMD (Totan et al., 2009). Concurrently, studies have shown increased oxidative stress in the retina from donors' eyes with AMD (Ethen et al., 2007; Golestaneh et al., 2017; Renganathan et al., 2008; Terluk et al., 2015). Cultured RPE cells from AMD donors' eyes revealed higher ROS production and malfunctioning of the mitochondria (Golestaneh et al., 2017). Oxidative damage to mitochondrial and nuclear DNAs was observed in the RPE of AMD subjects

(Blasiak et al., 2013; Terluk et al., 2015). Donor eyes with AMD showed a higher concentration of the carboxyethylpyrrole (CEP) content in the BrM compared to normal eyes (Crabb et al., 2002). CEP is a lipid peroxidation product formed from docosahexaenoic acid (DHA) under oxidative stress (Liang Lu et al., 2009). Since the photoreceptor outer segments are largely composed of DHA (Jeffrey et al., 2001), the relative increase of CEP in the BrM from donor's eyes with AMD could suggest increased vulnerability to oxidative damage and/or greater exposure to oxidative stress compared to the non-AMD retina. Also, an accumulation of damaged proteins and impairment of autophagy, which is a proteolytic mechanism of efficient antioxidant capability, were observed in AMD (Mitter et al., 2014). In addition, the finding of cigarette smoking as a major risk factor of AMD in most epidemiological studies emphasized the crucial role of oxidative stress in the development of this retinal disease (Thornton et al., 2005; Velilla et al., 2013). The relationship between cigarette smoking and AMD has been demonstrated experimentally in vitro and in wild-type mice and was shown to be directly linked to the oxidants in cigarette smoke (Espinosa-Heidmann et al., 2006; A L Wang et al., 2009). The cigarette smoke extract (CSE) was observed to increase lipid peroxidation by 8-fold in RPE (Kunchithapautham et al., 2014). Investigating the neuroprotection of antioxidants in the development of AMD may be helpful to further entrench the significant role of oxidative stress in AMD as existing literature is inconclusive (J R Evans & Henshaw, 2008; Sin et al., 2013). Next, we provide a comprehensive review of the animal models to provide insight into the involvement of oxidative stress in the development of AMD.

Cigarette-smoke model

The exposure of mice to cigarette smoke is an important way to investigate the role of oxidative stress in AMD because cigarette smoking is the most significant modifiable

risk factor in AMD (Armstrong and Mousavi, 2015; Velilla et al., 2013). Cigarette smoke contains several potent oxidants, including hydroquinone (HQ), nicotine and cadmium (Horinouchi et al., 2016; Leanderson & Tagesson, 1990). This animal model has helped to understand possible molecular events that might lead to AMD. HQ upon entry into the circulation through the lungs diffuses into cells and affects the mitochondria, resulting in the overproduction of superoxide anion and damage to mitochondrial membranes (Espinosa-Heidmann et al., 2006). Leakage of superoxide into the cytoplasm generates ROS, which mediates protein oxidation and lipid peroxidation. In addition, complement activation was found in the cigarette-smoke mediated retinal degeneration (Kunchithapautham et al., 2014; Woodell & Rohrer, 2014).

Espinosa-Heidmann and colleagues demonstrated that HQ from cigarette smoke could cause subretinal deposits, a hallmark of AMD (Espinosa-Heidmann et al., 2006). The protocol for inducement normally involves repeated daily exposure of adult mice to cigarette smoke pumped into sealed chambers for a certain time period of the day. Exposed mice showed elevated serum HQ levels and oxidative stress accompanied by retinal changes including BrM deposits and thickening, inflammation and choroidal neovascularization (Espinosa-Heidmann et al., 2006; Masashi Fujihara et al., 2008; A L Wang et al., 2009). This rodent model shows that oxidative stress could induce other molecular mechanisms to generate the AMD phenotype. The severity of the retinal degeneration was shown to depend on the length of exposure. In a study where mice were exposed to cigarette smoke for 2 hours daily for 4.5 months, there was less damage of the RPE compared to the result from another study which used a 5-hr daily exposure for 6 months (Espinosa-Heidmann et al., 2006; Masashi Fujihara et al., 2008). The

extended exposure time to cigarette smoke, led to apoptosis of the RPE (Masashi Fujihara et al., 2008).

Light-induced model

The damaging effect of light on the retina has been studied and reported to affect mainly the outer retina and RPE (Hunter et al., 2012; Lanum, 1978; J. Wu et al., 2006). Intense retinal illumination has been associated with a reduction in the thickness of the outer nuclear layer, and accumulation of deposits within the RPE (Organisciak & Vaughan, 2010). Most studies of retinal damage by light have used the BALB/c mice or SD rats, known for their genetic susceptibility to light. Recently, a novel protocol by which retinal degeneration could be induced in the commonly available C57BL/6J mice was described (Zhong et al., 2016). The wide use of the light-induced retinal degeneration model for studying AMD is because it offers advantages such as synchronized photoreceptor death occurring with light exposure and is easy to induce within a short time (Wenzel et al., 2005). Also, this model offers the possibility to vary the severity through manipulation of light intensity and duration, and more importantly because light is a natural risk factor involved in many retinal diseases (Organisciak & Vaughan, 2010).

One of the mechanisms accounting for the retinal damage is due to the interaction between light and photosensitive molecules such as rhodopsin and lipofuscin (Rózanowska & Sarna, 2005; J. Wu et al., 2006). The activation of rhodopsin coupled with another cascade of events in the phototransduction process has been associated with photoreceptor cell death (Rózanowska & Sarna, 2005; Wenzel et al., 2005). It is likely that such rhodopsin-mediated mechanism is related to oxidative stress because antioxidant interventions have been found to preserve photoreceptor (Fang et al., 2016;

Rozanowska, 2012; Tomita et al., 2005). In addition, light causes the formation of lipid peroxidation from the DHA content of the rods outer segment membranes (Catalá, 2006; Pasantes-Morales & Cruz, 1985). The first light-induced model of retinal degeneration was demonstrated by Noell in albino rats (Noell et al., 1966). Since then, several researchers have used similar protocols which differ with respect to the presence of genetic susceptibility of the animal, intensity, wavelength, and duration of light exposure (Grimm & Remé, 2013; Hafezi et al., 2000). Shorter wavelengths closer to the blue region of the electromagnetic spectrum have been found to have the highest risk for retinal degeneration compared to the longer wavelengths (Wu et al. 2006). The susceptibility to retinal damage by the blue light is due to the increased generation of ROS by the photoactivated pigments rhodopsin and lipofuscin in response to this specific range of the electromagnetic spectrum (Boulton et al., 1993; Demontis et al., 2002).

Carboxyethyl pyrrole immunization model

The carboxyethyl pyrrole (CEP) model elucidates the mechanism by which oxidative damage to cellular molecules including lipids and proteins could result in inflammation and retinal degeneration. CEP modified protein adduct is generated by oxidative damage to DHA with subsequent reaction with the lysine moiety of adjacent proteins. CEP protein adducts found in AMD are immunogenic, inducing autoantibodies production and inflammation in the retina (Crabb et al., 2002; Gu et al., 2003). The immunization of mice with CEP-modified mouse serum albumin (CEP-MSA) induced antibodies against CEP and led to inflammatory responses such as the deposit of complement component-3 in BrM and macrophage infiltration (Hollyfield et al., 2008). Retinal changes observed after 12-14 months of single immunization included loss of

RPE, drusen formation and thickening of the BrM. These retinal degenerative changes could be induced within a shorter duration (i.e. 2-3 months) in mice through repeated immunization. This model may be useful in investigating mechanisms by which oxidative stress may mediate inflammation in AMD. Based on the outcome of the cigarette smoke, light damage and CEP immunization in mice's retina, the pathways by which oxidative stress may lead to AMD have been described (**Fig.** 2.1).

Dysregulated antioxidant mechanisms and AMD

Antioxidants and other antioxidant-related mechanisms play important roles in reducing oxidative stress. Antioxidants are intracellular molecules that scavenge reactive oxygen species (ROS) and enzymes which degrade superoxides and hydroperoxides, protecting against oxidative stress (Hosaka et al., 2005). The vitamins (A, C and E) and carotenoids *(*lutein and zeaxanthin), potent and effective antioxidants, are essential in retinal function. Carotenoids, in particular, zeaxanthin, are found in the central retina constituting the macular pigment shown to be protective against light-induced oxidative damage through absorption of the near-blue wavelength of light (Loskutova et al., 2013). Vitamin E (α-tocopherol) may protect the retina from oxidative damage a by acting as a scavenger of lipid peroxyl radicals (Niki, 2014). Studies have shown that increased antioxidants in diet or serum could be protective against AMD progression. According to a longitudinal clinical study, dietary intake of antioxidant/zinc was found to reduce the risk of early AMD in a highly susceptible population due to genetic polymorphisms of complement factor H (CFH) Y402H and LOC387715 A69S (Ho et al., 2011). Although some studies found no protective effect of antioxidants against early AMD (Chong et al., 2007), there is little controversy over its protective role in late AMD (Michikawa et al., 2009). Also, experimental studies in

monkeys have found that the consumption of antioxidant-deficient diet (vitamins A and E, and B carotene deficiencies) was associated with photoreceptor degeneration and lipofuscin accumulation in the RPE (Malinow et al., 1980; Steele & O'Tousa, 1990).

In addition to the antioxidants, autophagy and nuclear factor erythroid 2-related factor 2 (NRF2) and their associated antioxidant enzymes have been found to be highly beneficial for retinal survival under both normal and adverse conditions (de Vries et al., 2008; Giordano et al., 2013). Autophagy is a cellular recycling mechanism possessing efficient antioxidant properties and protective against neurodegenerative diseases (Giordano et al., 2013). An age-related upregulation of autophagy occurs in the retina of non-AMD donor and mice indicated by an increase in the number of the autophagosome, autophagy-related proteins and autophagy flux was noted (Mitter et al., 2014). In contrast, donor eyes with AMD showed a decline in autophagy, suggesting it may be involved in the disease (Mitter et al., 2014; Yao et al., 2015). One master regulator of the cellular antioxidant mechanism is NRF2, a transcription factor that regulates the production of antioxidant enzymes against oxidative stress. Under quiescent conditions, NRF2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytosol, inactive, and predestined for degradation by the ubiquitin-proteasome pathway (Wakabayashi et al., 2003). However, NRF2 unbinds from Keap1 under oxidative stress resulting in its upregulation and translocation into the nucleus. This leads to the upregulation of several antioxidant genes and enzymes including heme oxygenase 1 (HO-1), NAD(P)H-quinone oxidoreductase (NQO1), glutathione S-transferase (GST), superoxide dismutase (SOD), ferritin, and glutathione reductase. SOD is a ubiquitous family of enzymes present in all oxygen-metabolizing cells. It constitutes the first line of defense against superoxide radical (O_2^-**)** and other ROS (Matés et al., 1999). The O_2^- radical is a very potent oxidative agent because each free

radical rapidly gains three electrons to rebalance itself, unlike other ROS. Also, O_2^- could generate other ROS, particularly hydrogen peroxide and hydroxyl radicals.

In the retina, Cu-Zn (SOD1), Mn-SOD (SOD2), and Fe-SOD (SOD3) are found in the cytosol, mitochondrial matrix and tissue extracellular space, respectively (Behndig et al., 1998). Mice models of retinal degeneration induced by compromising the antioxidant mechanisms provide a platform to understand the role of oxidative stress in AMD.

Autophagy deficiency models

The protective role of autophagy against AMD has been demonstrated *in vivo* through the impairment of autophagy to observe any consequential changes in the retina (Yao et al., 2015; Y. Zhang et al., 2017). Yao and colleagues found that the deletion of the RPE gene encoding RB1CC1 (inducible coiled-coil 1) inhibited autophagy in mice's RPE and accompanied by age-dependent retinal changes including RPE atrophy, microglial infiltration, sub-RPE deposits, and CNV. Subsequently, photoreceptors degenerated and loss of retinal functions occurred (Yao et al., 2015). Similar results were observed in mice's retina following the deletion of RPE-specific Atg5 and Atg7 (Y. Zhang et al., 2017). Mice aged 8 months old developed retinal signs of early AMD such as abnormal RPE thickness and photoreceptor degeneration (Y. Zhang et al., 2017). This occurrence of oxidative damage in the retina following the inhibition of autophagy is an indication that autophagy could be an important antioxidant mechanism in the retina. In addition, impairment of autophagy is shown to induce inflammation through the recruitment of inflammasome-activated macrophages (Liu et al., 2016). Liu and co-workers showed that impairing autophagy in the eyes of mice via intravitreal injection of wortmannin, an autophagy inhibitor that irreversibly inhibits

class III PI3-Kinase12, transiently reduced autophagy activity for one week, and led to photoreceptor and RPE death by apoptosis (J. Liu et al., 2016a). These findings support the involvement of autophagy in the maintenance of normal homeostasis in the aging retina and its impairment may play a role in aging retinal degeneration

NRF2 deficiency model

Retinal degenerative changes, the presence of hard and soft drusen and RPE atrophy, are observed in adult NRF2 knockout mice by fundus examination (Z. Zhao et al., 2011). Retinal function assessment revealed a decline in the a-wave and b-wave amplitudes in electroretinograms (ERGs). Microscopic examination of their retinas showed thickening of the BrM and sub-RPE deposits comprising of complement components C3d and vitronectin, which are indicators of complement pathway activation. In some eyes, CNV lesions and loss of photoreceptors were also observed. The age-dependent retinal damage occurring in NRF2-deficient mice elucidates the importance of dysregulated antioxidant mechanisms and oxidative stress in the development of AMD. According to Zhao and coworkers, there was additional downregulation of autophagy in the NRF2-deficient mice, which they described as an increase in autophagosome and autolysosome and accumulation of oxidatively damaged protein aggregates and organelles (Z. Zhao et al., 2011). However, the impact of NRF2 deficiency on autophagy inhibition may require further investigation to be established since increased autophagosome and autolysosome could as well be indications of upregulated autophagy when the stage of binding to these to the lysosomes is uncompromised (A. Jain et al., 2015; Komatsu et al., 2010). Even though this NRF2 deficiency model may be helpful in addressing some questions in AMD, its use is limited by the fact that the role of NRF2 in AMD remains unclear. It is yet to be

demonstrated whether NRF2 is differentially expressed in AMD donor eyes compared to normal healthy eyes. However, recent experimental data in mice have also supported the association between NRF2 deficiency and AMD, as it was found that the NRF2 mRNA expression under oxidative stress was impaired in the RPE of aged mice compared to younger mice, thus making this association probable in humans (Sachdeva et al., 2014).

SOD-deficiency model

The retinal changes occurring in SOD1- and SOD2 knockout mice emphasize a major role of oxidative stress in the pathogenesis of AMD. *Sod1*$^{-/-}$ aged 10 months or older developed retinal drusen, thickened BrM, and CNV. In addition, degenerative changes were found in the RPE and photoreceptors of some mice (Imamura et al., 2006). Sandbach and colleagues demonstrated that the deficiency in SOD2 expression was associated with an increased mitochondrial ROS production (Sandbach et al., 2001). SOD2 deficiency is lethal and SOD2 knockout mice die within one week from systemic abnormalities related to oxidative damage (Y. Li et al., 1995; Melov et al., 1999). Hence, to study retinal changes arising due to SOD2 deficiency, these mice were treated with the SOD2 mimetic, manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP), which extended their lifespan to 3 weeks. MnTBAP-treated SOD2 knockout mice showed thinning of the inner retinal layer and photoreceptor layer compared to wild-type. No change was observed in the RPE and BrM. Perhaps either the short lifespan of SOD2 knockout mice does not allow sufficient time for additional age-related retinal abnormalities to develop or the MnTBAP treatment for the SOD2 knock out mice have protective properties.

Also, other researchers investigated retinal damage occurring in SOD2 deficiency using a gene therapy approach to overcome the problem of lethality with SOD2 knockout

mice (Justilien et al., 2007). The adeno-associated virus (AAV) expressing a ribozyme gene (Rz432) was administered by subretinal injection into the eyes of adult C57BL/6 mice to target the RPE and knockdown SOD2 expression in adult mice (Qi et al., 2003). Eyes treated with Rz432 had reduced SOD2 proteins and increased oxidative damage. At 4 months post-treatment, retinal changes typical of human AMD such as the loss of a- and b-waves amplitudes of ERG, accumulation of oxidized proteins in RPE and degeneration, thickening of BrM, apoptotic photoreceptor death, and increased deposit of A2E, the lipofuscin fluorophore, were observed in most treated eyes (Justilien et al., 2007). However, the role of SOD in human AMD remains uncertain since an earlier genetic study reporting an association between SOD polymorphism and AMD (Kimura et al., 2000) has recently been challenged (Kan et al., 2014). Also, results from a previous study investigating SOD enzyme levels in RPE from donor's eyes with or without macular degeneration found no significant correlations between SOD and aging or macular degeneration (Liles et al., 1991), making this mechanism only speculative.

α-Tocopherol deficiency model

α-Tocopherol (vitamin E) is a potent fat-soluble antioxidant known for its role as a scavenger of lipid peroxyl radicals. The physiological role of α-tocopherol in the body is evident from systemic conditions such as neurological dysfunction, myopathies, and diminished erythrocyte lifespan associated with its deficiency. Following absorption, α-tocopherol is transported to parenchymal cells of the liver for storage (Jensen et al., 2006). The serum concentration of α-tocopherol is regulated by *α*-tocopherol transfer protein (α-TTP) which is involved in its transport from the liver to other body organs (Kono et al., 2013). Mutations in the gene encoding *α*-TTP are linked to ataxia with isolated vitamin E deficiency (Kono et al., 2013; Ouahchi et al., 1995).

Since the retina has a rich lipid content, it was thought that α-tocopherol could have an impact. Therefore, to investigate the protective effect of vitamin E on oxidative stress, α-TTP knock out mice were generated and fed vitamin E deficient diet (Yokota et al., 2001). There was increased lipid peroxidation and degeneration of neurons and α-TTP deficient mice showed changes in retinal function indicated by attenuation in a- and b-wave in ERG at 12 months, as well as loss of outer and inner segments of photoreceptors by 20 months. This outcome shows that α-tocopherol is a potent antioxidant in the retina protective against oxidative stress-related retinal degeneration. However, long-term clinical trials investigating the neuroprotection of vitamin E supplement intake on the development or progression of AMD have consistently found no significant clinical effect (Christen et al., 2011; Taylor et al., 2002). Since vitamin E deficiency in humans is rare and is often found in isolated cases of abnormal dietary fat absorption or metabolism rather than from a diet low in vitamin E (Manor & Morley, 2007; Muller et al., 1983), that could explain why its intake as supplement may not be of additional benefit in persons with AMD having normal metabolism.

Inflammation and AMD

Numerous reports have discussed the roles of inflammation in AMD pathogenesis extensively (Jayakrishna Ambati et al., 2013; Kauppinen et al., 2016). Analysis of drusen from donated eyes with AMD revealed the presence of complement components C3 and C5 and membrane-attack-complex (MAC), suggesting the activation of the complement pathways. In addition, regulators of the complement pathways such as vitronectin and clusterin inhibiting the pathways are also present in drusen (Anderson et al., 2002; Crabb et al., 2002; Donoso et al., 2006; L V Johnson et al., 2001). Activation of the complement system causes proinflammatory responses such as the

production of membrane-attack complexes (MAC), leading to cell lysis and release of chemokines to mediate recruitment of inflammatory cells including microglia and macrophages (Donoso et al., 2006; Gemenetzi & Lotery, 2015). The role of inflammation in the pathogenesis of AMD is solidified by genetic studies showing that the Y402H polymorphism of the complement factor H (CFH), a soluble glycoprotein regulating complement activation, is found in more than half of AMD cases and that the presence of this polymorphism is associated with a higher risk of this disease (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005; Lin et al., 2005). Other indicators of inflammation in AMD are the presence of chemokines and the accumulation of immune cells such as macrophages and microglia in the retina of AMD subjects (Killingsworth et al., 1990; Skeie & Mullins, 2009). Also, the ability to create animal models of retinal degeneration through manipulation of the immune response further highlights the importance of inflammation in AMD. Various animal models supporting inflammation in AMD are discussed in this review.

Chemokine models

Chemokines and their G-protein coupled receptors contribute significantly to inflammation in AMD. The role of chemokines in AMD is evident from the increased infiltration of activated macrophages and microglia in the milieu of drusen and atrophic lesions (L V Johnson et al., 2001; Killingsworth et al., 1990). The enhanced recruitment of these immune cells is due to the differential expressions of chemokines and its receptors in AMD (Falk et al., 2014; Grunin et al., 2012). Chemokines are grouped into four families depending on their conserved cysteine residues: CXC, CX3C, CC, and C (Laing & Secombes, 2004; Lira & Furtado, 2012). CCL2/CCR2 and

CX3CL1/CX3CR1, which are ligand/receptor pairs have been implicated in macrophages and microglia recruitment, respectively in AMD (Raoul et al., 2010).

$Ccl2^{-/-}$ or $Ccr2^{-/-}$mice model

This model presumed that the recruitment of macrophages into the retina was protective. Ambati et al showed that knocking out *Ccl2/ Ccr2*, a chemokine receptor expressed by macrophages and its binding molecule, respectively, in mice led to the inhibition of macrophage recruitment and retinal degeneration. Signs of retinal degeneration found in the adult (16 months and older) mice's retina were drusen, lipofuscin, thickening of the BrM and geographic atrophy or CNV (Ambati et al., 2003). These findings, however, have been challenged by others who demonstrated that $Ccl2^{-/-}$ or $Ccr2^{-/-}$mice showed no change in the thickness of either the BrM or RPE, as well as not affecting the photoreceptors (Luhmann et al., 2009, 2013). Intriguingly, the same controversy on the involvement of *CCR2/CCL2 in AMD is found in studies using human participants too*. A case-control study of the association between *CCL2*/*CCR2* polymorphisms and AMD showed no significant association between these genes and AMD (Despriet et al., 2008). Furthermore, quantitative PCR reactions evaluating the expression of these genes in laser-dissected RPE from 13 donor AMD and 13 control eyes found no significant difference in the expression of these genes between normal subjects and those with AMD. Controversially, another case-control study with relatively fewer subjects subsequently reported a significant difference in the genotype and allele frequency for *CCL2/CCR2* between AMD and normal controls and concluded that individuals possessing both single nucleotide polymorphisms (SNPs) were at a higher risk of developing AMD (Anand et al., 2012). More recently, the

demonstration that different functional macrophage subtypes may exist (either protective or injurious) may help to resolve the controversy (Zandi et al., 2015). Mice given an intravitreal injection of M2 macrophages, a subtype accumulating in wet AMD, displayed exacerbated CNV lesions while mice injected with M1 macrophages displayed ameliorated CNV lesions (Zandi et al., 2015). The exact role of macrophages and their receptor-ligand pairs (CCR2/ CCL2) in AMD require further investigation.

Cx3cr1$^{-/-}$ mice model

On the contrary to the proposal that infiltration of macrophages into the retina is protective, the *Cx3cr1$^{-/-}$*mice model suggests an accumulation of microglia, CNS-resident macrophages, in the retina is harmful (Combadière et al., 2007). An in-vitro study showed that microglial cells induced the death of photoreceptor cells (Roque et al., 1999). Microglia are the first and main form of active immune defense in the central nervous system (Olson & Miller, 2004). Combadiere and colleagues demonstrated that *Cx3cr1$^{-/-}$* mice had impaired microglial egress from the retina, resulting in its accumulation (Combadière et al., 2007). The *Cx3cr1$^{-/-}$*mice developed sub-RPE deposits, photoreceptor degeneration, and CNV. Recently a meta-analysis of findings from five long-term studies, which suggested no association between common *CX3CR1* variants and AMD (Schaumberg et al., 2014). Again, no agreement has been reached on the role of CX3CR1 in human population studies.

CCL2$^{-/-}$ /CX3CR1$^{-/-}$ double knockout model

The *Ccl2$^{-/-}$ /Cx3cr1$^{-/-}$* double knockout murine model was employed to overcome the shortcomings of the longer average time taken for either *Ccl2−/− or Cx3cr1$^{-/-}$* single knockout mice to express AMD phenotype. The researchers reported success in the

creation of a murine model that took between 4–6 weeks to exhibit visible drusen-like lesions, as well as histological signs of AMD including thickening of BrM, localized hypopigmentation, and degeneration of RPE, and photoreceptor atrophy (Chan et al., 2008; Tuo et al., 2007). CNV was also observed in some of the mice. In addition, there was an increased deposition of lipofuscin granules and its component N-retinylidene–N- retinyl ethanolamine (A2E) in the retina of these mice. Also, signs of active inflammation were found in the double knockout mice model, including complement C3, macrophages and activated microglia (Ross et al., 2008). However, the reproducibility of this murine AMD model is doubtful as works by others have challenged that there could be some other genetic mutation in the breeding pair of mice used (Raoul et al., 2010). Assuming that the outcomes reported in the different chemokine-models were valid, then inflammation may be crucial in the development of CNV and AMD.

Complement activation models

Even though several reports pointed to activation of the complement pathways in the AMD retina, (Anderson et al., 2002; L V Johnson et al., 2001), the discovery that polymorphisms in the gene for CFH were associated with greater susceptibility to AMD demonstrates the contribution of this mechanism to the pathogenesis of this disease (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005; Lin et al., 2005). One of the reasons for the susceptibility of the Y402H polymorphism of *CHF* in AMD is due to the impaired binding of CFH to the BrM resulting in unregulated complement activation and chronic local inflammation (Clark et al., 2010). CFH regulates the complement system by inhibition of alternative pathway through direct inactivation of C3b or binding to C3b, ultimately inhibiting the synthesis of C3 convertase (Ding et

al., 2015). Depletion of C3 convertase is necessary otherwise it could lead to MAC formation causing lysis of RPE. CFH reaches the retina mainly by circulation, although some amount is also synthesized by the RPE (Chen, Forrester, & Xu, 2007; Kim et al., 2009). Apart from the CFH gene, polymorphisms in *C3* have also been found to be associated with increased susceptibility to AMD (Maller et al., 2007; Yates et al., 2007). Meanwhile, polymorphisms in complement factor B and complement components 2 (C2) have been found to be protective against AMD (Gold et al., 2006; Hughes et al., 2011).

Models of complement factor H deficiency

The role of CFH in inflammation and AMD is supported by the retinal degenerative changes observed in the $Cfh^{-/-}$ mice and $Cfh^{+/-}$ mice. Older surviving *Cfh-/-* mice developed characteristic AMD signs including visual functions and the accumulation of subretinal deposits (Coffey et al., 2007; Toomey et al., 2015). Similarly, adult *Cfh+/-* mice fed on high cholesterol diet developed similar functional and structural changes (Toomey et al., 2015). These models elucidated the pathophysiological roles of CFH in the retina by showing that the increased sub-RPE deposits in the $Cfh^{+/-}$ and $Cfh^{-/-}$ mice was due to the competition between CFH and lipoprotein for binding to the BrM. $Cfh^{+/-}$ and $Cfh^{-/-}$ mice, having a deficiency in CFH expression, had lipoprotein accumulation in the BrM forming sub-RPE deposits. They further demonstrated that the $Cfh^{+/-}$ mice showed dysregulated activation of complement in the BrM causing inflammation. However, a systemic difference exists between $Cfh^{-/-}$ mice and *CFH* polymorphism. CFH knockout mice have decreased plasma C3 concentration, unlike *CFH* polymorphisms which do not present with changes in C3 levels (Coffey et al., 2007).

Humanized CFH mice

This transgenic murine model was developed to evaluate the importance of Y402H polymorphism in AMD. Obviously, a mouse model of AMD based on the Y402H variants, the commonest genetic risk factor found in AMD, would help elucidate the pathological mechanisms of this human disease. To create the Y402H *CFH* transgenic mouse, zygotes from wild-type mice were injected with plasmids containing Y402H variants of human *CFH* to substitute the mouse *Cfh* (Ufret-Vincenty et al., 2010). Matured transgenic mice aged 12 to 14 months showed drusen-like deposits in the central retina. There was an accumulation of macrophage and microglia, basal laminar deposits, and lipofuscin granules in the retina of these mice. In addition, there were signs of complement activation and inflammation in the retina. The inflammation may be brought about by the reduction in the affinity of CFH to bind to MDA, a lipid peroxidation product arising from oxidative stress in the retina. As such, the free MDA molecules could bind and activate macrophages resulting in inflammation (Weismann et al., 2011). It is possible that the Y402H polymorphisms lessen the efficiency of CFH to deal with oxidative stress making the aging retina vulnerable to AMD. However, there are conflicting results on the suitability of the humanized *CFH* mice as a model of AMD. Ding et al. generated humanized CFH mice by crossing transgenic mice having a full-length human *CFH* bacterial artificial chromosomes with $Cfh^{-/-}$ mice (Ding et al., 2015). Normal retinal morphology and function were preserved in those humanized *CFH* mice. In fact, even in humans, not all Y402H variants develop AMD. How Y402H polymorphism contributes to AMD remains to be examined.

C3-overexpressing mice

The retina from AMD eyes has increased complement expression and activation compared to normal eyes (L V Johnson et al., 2001). Therefore, to study whether increased complement activation underlies AMD, adult wild-type mice were administered subretinal injections of murine *C3*-carrying recombinant adenovirus (Cashman et al., 2011). C3 is a common converging point for the complement pathways, and its breakdown into C3a and C3b initiates the final process leading to the formation of MAC. Scotopic electroretinography showed functional deficits in these exogenous C3-overexpressing mice within 2 weeks. Histology and immunohistochemistry revealed pathological signs including RPE atrophy, loss of photoreceptor outer segments, reactive gliosis and retinal detachment. The deposition of MAC was observed in the outer segments of photoreceptors. While this model corroborates the role of the complement activation in AMD, its challenge is dealing with the involvement of adenoviruses themselves in the retinal pathological changes. Other than the surgical skill required, it could be a model of choice for investigating therapeutic interventions targeting the complement pathway due to the comparatively shorter duration required for creating this model.

Lipid metabolism and AMD

AMD is characterized by the accumulation of sub-RPE deposits including drusen, basal linear and basal laminar deposits, which are largely composed of lipid (C. a Curcio et al., 2011; Kishan et al., 2011). Another evidence supporting dysfunctional lipid metabolism in AMD is based on the association between aging and the accumulation of lipoproteins in the BrM. Lipoprotein accumulation could lead to the formation of a lipid wall, impairing the exchange of nutrients between choriocapillaris and RPE across the BrM, and compromising retinal functions (Curcio et al., 2011). Interestingly, the

location of this lipid wall is the same as the sub-RPE deposits found in AMD, possibly, it might be the precursor of these deposits. Lipoproteins found in the retina are either produced locally by the RPE or come from circulation (Cankova et al., 2011). Lipoproteins transport cholesterol across the BrM to/from the RPE and photoreceptors. Apolipoprotein is the protein constituent found in lipoproteins. Other results implicating the role of lipid metabolism in AMD comes from the association between apolipoproteins and AMD (Thakkinstian et al., 2006). APOE and APOB of low-density lipoproteins (LDL) facilitate lipid metabolism through binding to specific receptors on the liver and other cells. *APOE4* polymorphism is protective against AMD whereas *APOE2* polymorphism is associated with increased risk of AMD (Souied et al., 1998; Thakkinstian et al., 2006). The protective role of APOE4 has been linked to its increased receptor-binding affinity as compared with APOE2. Thus, APOE4 may facilitate greater lipid metabolism due to its increased binding affinity to the liver, the primary site of lipid metabolism. Others have also reported that APOE4 is associated with higher macular pigment optical density, which also might confer protection against AMD (Loane et al., 2010). Also, an association between atherosclerosis and AMD has been reported (Tan et al., 2007). Based on the fact that increased serum cholesterol-lipoprotein is a hallmark of atherosclerosis, then by extension, it implicates increased cholesterol in AMD (Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group, 1990). A direct association between AMD and increased serum cholesterol has been supported by a population-based study showing that higher serum HDL concentration in aged persons doubled their risk of developing AMD (Cougnard-Grégoire et al., 2014). Also, the contribution of dysfunctional lipid metabolism to AMD is upheld by the finding that an intervention modulating lipid metabolism was effective in managing AMD (Vavvas et al., 2016). In this follow-up

study of 23 subjects diagnosed with AMD having large soft drusen, a high dose of atorvastatin treatment resulted in a regression of the drusen and vision gain in 10 patients. No subject progressed to advanced neovascular AMD. In addition, the induction of retinal degeneration in animals by the manipulation of genotypes responsible for lipid metabolism further supports the involvement of this mechanism in AMD.

Humanized apolipoprotein and Apoe$^{-/-}$ mice models

Researchers have demonstrated an association between dysfunctional lipid metabolism and AMD in mice through the expression of variant apolipoproteins. It was shown that adult humanized transgenic mice expressing one of the three human APOE isoforms (APOE2, APOE3, OR APOE4) and fed high cholesterol-containing diets for 8 weeks developed age-related retinal degenerative changes (Malek et al., 2005). However, the results sharply contrast the findings from human genome studies which show that APOE4 is protective against AMD (Fritsche et al., 2009; Thakkinstian et al., 2006). Also, other models such as *Apoe*−/− mice, humanized *APO*E3-Leiden* mice, and humanized *APOB100* mice support a relationship between hypercholesterolemia and AMD (Dithmar et al., 2000; Kliffen et al., 2000; Goldis Malek et al., 2003). All these adult transgenic mice when fed high-fat diets showed hyperlipidemia accompanied by thickening of the BrM and subretinal deposit resembling basal linear deposits which were all age-related except for the Apoe$^{-/-}$ mice (Dithmar et al., 2000). In humans, the APO*E3-Leiden, a defective human APOE-3 variant, was associated with hyperlipoproteinemia (i.e. inability to break down cholesterol and triglycerides) and early onset atherosclerosis (Gijbels et al., 1999; L. E. Viiri et al., 2008). Apolipoprotein B100 (APOB100) is another type of low-density lipoprotein (LDL) involved in

cholesterol transport. It is one of the components in sub-RPE deposits in AMD (Goldis Malek et al., 2003). Several researchers have studied the role of APOB100 in AMD and most have directly linked the development of retinal degeneration to increased cholesterol accumulation in the retina (Espinosa-Heidmann et al., 2004; Masashi Fujihara et al., 2009; Sallo et al., 2009). However, Espinosa-Heidmann and colleagues have contested that there were no obvious increased cholesterol deposits in the BrM and suggested that it is solely due to hyperlipidemia in the APOB100 mice (Espinosa-Heidmann et al., 2004). They showed that the retinal degeneration occurred much faster in younger humanized ApoB100 mice fed a high-fat diet following exposure to blue-green light, suggesting the importance of lipid peroxidation. Furthermore, it was shown that prophylactic treatment of APOB100 mice with subcutaneous injection of the antioxidant tocopherol prevented retinal degeneration in the APOB100 mice. These together reveal that a higher risk of lipid peroxidation in the retina may be a plausible mechanism by which hyperlipidemia may be associated with retinal damage.

Cd36$^{-/-}$ *mice*

The *Cd36*$^{-/-}$ mice model shows the significance of phagocytosis and breakdown of photoreceptor outer segment (POS) by the RPE as a mechanism for maintaining normal retinal homeostasis. CD36, also referred to as fatty acid translocase (FAT), is a membrane glycoprotein used by cells for recognition and binding to specifically oxidized low-density lipoproteins, oxidized phospholipids, and long-chain fatty acids, for transport into cells (Bonen et al., 2004; Ibrahimi & Abumrad, 2002). In the retina, CD36 is abundantly expressed by the RPE and involved in the recognition and phagocytosing of oxidized POS (Picard et al., 2010; Ryeom et al., 1996). *Cd36* knockout mice, therefore, have an accumulation of oxidized LDL, thickening of BrM

and photoreceptor death (Picard et al., 2010). Hence, this model, like the apolipoprotein and *Apoe*$^{-/-}$ mice, supports increased lipid peroxidation in AMD.

Ldl receptor$^{-/-}$ *and Vldl receptor*$^{-/-}$ *mice*

Findings from the *Ldl* receptor$^{-/-}$ and *Vldl* receptor$^{-/-}$ mice enhance our understanding of the association between AMD and dysfunctional lipid metabolism by showing that the lack of LDL/VLDL in mice resulted in changes in the vascular endothelial growth factor (VEGF) expression and retinal neovascularization (Hu et al., 2008; Rudolf et al., 2005). The LDL receptors bind to APOB- and APOE-containing lipoproteins to facilitate lipid metabolism (Law & Scott, 1990; Mahley, 1988). These receptors are abundantly expressed by the liver, to aid in the uptake of cholesterol (Brown & Goldstein, 1983). Similar to the variant humanized apolipoprotein mice and Apoe$^{-/-}$ mice, mice lacking LDL receptors had increased plasma cholesterol due to impaired cholesterol metabolism (Rudolf et al., 2004). Thickening of the BrM was observed in the *Ldl* receptor$^{-/-}$ mice fed a high-fat diet. In addition, these mice expressed increased levels of VEGF in the outer retinal layers (Rudolf et al., 2005). The VLDL receptors are also involved in the binding and uptake of ApoE-containing lipoproteins. VLDL receptors are expressed in the retinal vascular endothelium and RPE of mice (Hu et al., 2008). Interestingly, mice lacking VLDL receptors showed normal serum cholesterol levels but developed retinal degeneration characterized by neovascularization at 2 weeks postnatal, followed by photoreceptor degeneration, RPE hyperplasia, and subretinal fibrosis at the end-stage (Heckenlively et al., 2003; Hu et al., 2008). The results of these mice models support LDL/VLDL receptors as negative regulators of retinal neovascularization and interventions targeting them might prove beneficial in

the management of wet AMD. The Wnt pathway has been proposed as the mechanism by which VLDL receptors modulate VEGF expression and neovascularization (Chen, Hu, Lu, Flannery, & Ma, 2007).

LDL injection model in rats

This model supports a relationship between hyperlipidemia and AMD by showing that repeated intravenous injections of LDL for 7 days resulted in the accumulation of apolipoprotein B100 in BrM, as well early AMD-like retinal changes including thickening of BrM, the death of photoreceptors, and inflammation in rat retina (Yin et al., 2012).

Angiogenesis and AMD

Angiogenesis describes the formation of new blood vessels from existing blood vessels by either splitting or sprouting (Risau, 1997). It is essential in development, reproduction, and repair., Dysregulated angiogenesis underlies several human diseases (Carmeliet, 2003). Progression of AMD may be associated with the development of new choroidal blood vessels into the central retina, an indication of dysregulated angiogenesis (Moult et al., 2014). This wet form of AMD characterized by CNV is a major cause of blindness in the elderly. Results from large population-based studies suggest that some populations may be more prone to wet AMD (Kawasaki et al., 2010). The formation of new capillaries involves a cascade of events beginning with the degradation of the underlying basement membrane of the existing blood vessel, by the proteolytic activity of the plasminogen activator system and matrix metalloproteinases (Pepper, 2001). This is followed by chemotactic migration and proliferation of

endothelial cells into the extracellular matrix stroma, formation of lumen and maturation of the endothelium (Liekens et al., 2001; Risau, 1997).

Most evidence implicates VEGF as the proangiogenic factor underlying CNV (Campa et al., 2010; Penn et al., 2008; A. Witmer, 2003). These are: (1) vitreous VEGF levels were found to be significantly higher in patients with AMD and CNV compared to healthy controls (Wells et al., 1996); and (2) clinical trials involving the administration of the anti-VEGF agents, including ranibizumab, bevacizumab, aflibercept, and pegaptanib, markedly suppressed neovascularization and vascular permeability in humans, and sustained gain of vision in many AMD patients (Schmidt-Erfurth et al., 2014). However, since VEGF is synthesized *in situ* by the RPE under both normal physiological conditions, it is argued that an equally potent inhibitory regulator must be involved in maintaining homeostasis in the normal retina (Dawson, 1999; Ohno-Matsui et al., 2001; Tong & Yao, 2006). One anti-angiogenic regulator synthesized by RPE is pigment epithelium-derived factor (PEDF). All the regulators are tightly controlled under normal physiological conditions. Conditions such as hypoxia, ischemia, and inflammation promote neovascularization by tilting the balance in favor of increased VEGF expression (Bressler, 2009; P A Campochiaro, 2000).

In mammals, VEGF binds to 3 VEGF receptors which differ in their affinity and function (VEGFR-1, VEGFR-2, and VEGF-3). The binding of VEGF to VEGFR-2 induces vascular permeability and angiogenesis in vivo (Gille et al., 2001). Binding of VEGF to VEGF-R1 may not produce an effect in itself, but VEGFR-1 negatively regulates activation of VEGFR-2 by acting as a decoy receptor which competitively

binds to VEGF, thereby modulating the amount of VEGF that binds to VEGFR-2 (Rahimi et al., 2000; Zeng et al., 2001). VEGFR-3, previously thought to be limited to angiogenesis in the embryo, has been found to be expressed on quiescent vascular endothelial cells in capillaries and is imputed for the induction of human gliomas and colon carcinomas (Witmer et al., 2001; Antonella N Witmer, Dai, Weich, Vrensen, & Schlingemann, 2002). Further details on the affinity of VEGF receptors to the subtypes of VEGF are comprehensively reviewed by others (Carmeliet & Jain, 2011).

Laser-induced CNV model

CNV was accidentally discovered as a complication of argon laser photocoagulation treatment of the eye. It was later demonstrated experimentally in monkeys that laser trauma resulting in rupture of the BrM could induce CNV (Ryan, 1979). A CNV-inducement protocol has been described for mice to allow repeatability of outcome (V. Lambert et al., 2013). Inflammation occurs following the rupture of BrM by laser trauma which may instigate increased VEGF expression in the retina (Krause et al., 2014; Paulus et al., 2015). Also, Berglin and coworkers demonstrated the involvement of matrix metalloproteinase-2 (MMP-2) in laser-included CNV (Berglin et al., 2003). They compared the size of CNV lesions created by laser trauma between mice lacking MMP-2 and normal wild-type mice and showed that laser exposure altered the MMP-2 gene and protein expressions resulting in bigger CNV lesions in wild-type mice than the MMP-2 knockout mice. The laser-induced CNV model has helped in the understanding of CNV and led to the development of effective therapy for CNV in human AMD. However, its usefulness is limited due to the substantial damage to the neural retina and BrM by laser treatment.

Light-induced CNV

Light-induced CNV is a useful model supporting oxidative stress as an upstream mechanism leading to angiogenesis (Albert et al., 2011). Oxidative stress enhances the expression of VEGF and PEDF in RPE, suggested to be protective against oxidative damage (Byeon et al., 2010; He et al., 2014). Albert and colleagues found that repeated exposure of albino rats to intense light for 12 hrs daily for 1 month resulted in an increased level of retinal lipid peroxidation product, and retinal changes including RPE and photoreceptor degeneration, sub-RPE deposits and CNV. Six months of intense light exposure resulted in complete loss of the outer nuclear layer and appearance of vast areas of CNV in rats. The progressive retinal changes seen in this light-induced model resemble human AMD and may be appropriate for investigating the pathophysiology of AMD.

VEGF overexpression models

These models have been used to investigate the role of VEGF overexpression in CNV through either injection of adenoviral vectors expressing VEGF into the RPE or subretinal injection of microbeads containing RPE, the primary source of VEGF in the retina (Baffi et al., 2000; Schmack et al., 2009; Spilsbury et al., 2000). Spilsbury and co-workers showed that subretinal injection of an adenoviral vector expressing rat VEGF resulted in overexpression of the exogenous VEGF and was accompanied by CNV in the rat eye 2 weeks after injection (Spilsbury et al., 2000). At about 3 months there was a loss of RPE and photoreceptors. Similar results were observed by Baffi and co-workers, who used a sub-retinal injection of an adenoviral vector expressing the human *VEGF* gene in rats (Baffi et al., 2000). Early retinal changes observed at 4 weeks of post-injection included subretinal exudates and CNV. In addition, there was a

shortening of photoreceptor outer segments and reduction of the outer nuclear layer at overlying areas of neovascularization.

Oshima and co-workers, however, argued that increased expression of VEGF alone in vivo could not cause CNV based on the finding that transgenic mice with increased expression of VEGF in RPE had normal retina and choroid (Oshima et al., 2004). It was further demonstrated that when there was a subretinal injection of a gutless adenoviral vector expressing Ang2, CNV consistently occurred. According to them, VEGF or angiopoietin 2 (Ang2) could not reach the choriocapillaris to induce neovascularization because of the tight junctions between RPE cells. There is, however, breaking of this barrier during subretinal injections of the adenoviral vector or microbeads. Also, the adenoviral vectors themselves could induce inflammation (Liu & Muruve, 2003). As a result, Wang and colleagues proposed the subretinal injection of an adeno-associated viral vector (AAV) encoding human VEGF in rats (Wang et al., 2003). Unlike adenovirus, AAV causes little or no inflammatory response and, hence, not likely to contribute to CNV (Lowenstein et al., 2007). While these models provided understanding into the association between increased retinal VEGF and AMD, the compromise of the retinal-blood barrier resulting from the subretinal injection could trigger an inflammatory response and promote the growth of new blood vessels (Szade et al., 2015).

Matrigel injection model

Matrigel is a basement membrane extract that can be used for investigating the roles of different angiogenic substances in CNV (Baatout & Cheta, 1996). It is liquid at 4°C but

solidifies to form a plug following injection into tissues (Passaniti et al., 1992). Matrigel may be composed of structural proteins and growth factors of choice depending on the requirement of an experiment. Implanted Matrigel can also be easily removed from tissue for quantification of angiogenesis by immunohistochemistry or histology. Shen and colleagues demonstrated that the subretinal injection of Matrigel induced CNV and other signs of retinal degeneration in the eyes of mice (Shen et al., 2006). Histological examination conducted at week 4 post-injection revealed solid Matrigel located beneath the neuroretina and above BrM. At 12 weeks, RPE and photoreceptor degeneration, and various degrees of CNV were also observed. In addition, macrophage infiltration and mild inflammation were seen in some CNV lesions. However, the reported success rate for inducing CNV in mice was low ranging from 30-55%.

Conclusion and perspective

The rodent models of retinal degeneration provide valuable evidence for the mechanisms involved in AMD and, in addition, help elucidate how these mechanisms may be interrelated with each other (**Fig.** 2.2). Oxidative stress appears, however, to be the common link to all the molecular mechanisms. The role of oxidative stress is implicated in the inducement of inflammation in the Y402H polymorphism, which is considered the most important genetic risk of AMD. In the Y402H polymorphism, there is marked a reduction in the ability of the complement regulator CFH to bind to MDA, a lipid peroxidation product (Weismann et al., 2011), leading to uncontrolled MDA-induced inflammation. This is because the binding of MDA to resident macrophages results in the release of cytokines by the macrophages (Weismann et al., 2011), and CFH serves as a decoy to MDA from binding to the macrophages. Thus, the higher risk of AMD associated with Y402H polymorphism is related to an increased vulnerability

to oxidative stress in the retina. The role of hyperlipidemia in AMD development also appears to be through increased oxidative stress. Analyzing data from rodent models generated to express the human apolipoproteins shows that these mice showed signs of hyperlipidemia, oxidative stress, and retinal degeneration. The hyperlipidemia (due to impaired lipid metabolism) causes a block of retinal blood vessels and thickening of Bruch's membrane (BrM), lead to ischemia and hypoxia, and the induction of oxidative stress. Finally, photooxidative damage in mice's eyes has been shown to induce CNV. It is explained that increased oxidative stress promotes increased VEGF production by the RPE cells, as a response to counteract the harm from oxidative stress in the eye. The unregulated VEGF production, however, stimulates the development of new blood vessels. Therefore, targeting of VEGF or inflammation, which is the current focus for AMD treatment, although has proven to be an effective approach, may be addressing the late stages of this vision-threatening condition. The autophagy and NRF2 mechanisms are cellular defense mechanisms found to support survival under oxidative stress. The relationship between oxidative stress and autophagy or NRF2 is bidirectional; increased oxidative stress activates autophagy or NRF2, and these antioxidant defense mechanisms, in turn, could inhibit oxidative damage in the retina. Since impaired autophagy, NRF2, or dyslipidemia are those factors that could promote increased oxidative stress in the retina, we would implore future studies to target these pathways as alternative therapeutic options for AMD.

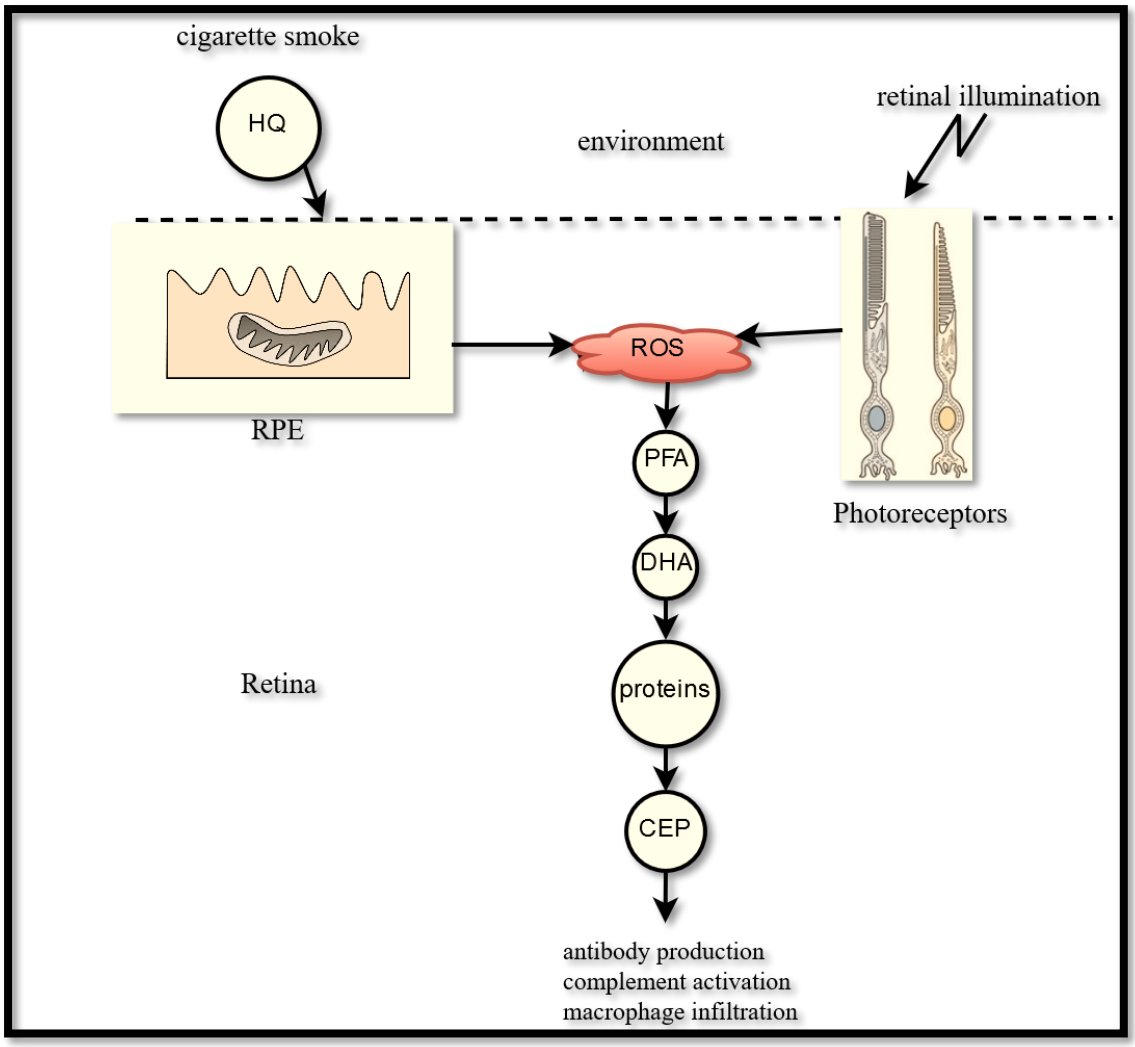


Figure 2. 1: Molecular mechanism in oxidative stress-induced retinal degeneration in mice.

Damage of mitochondria leads to the accumulation of reactive oxygen species (ROS) intracellularly in RPE and photoreceptors cells following (1) exposure to the cigarette smoke pro-oxidant, hydroquinone (HQ) or (2) photoactivation of rhodopsin by light. A chain of oxidative reactions results in the formation of the carboxyethylpyrrole (CEP) from the docosahexaenoic acid (DHA), a polyunsaturated fatty acid content (PFA) constituent in the retina. CEP is immunogenic, leading to an inflammatory response.

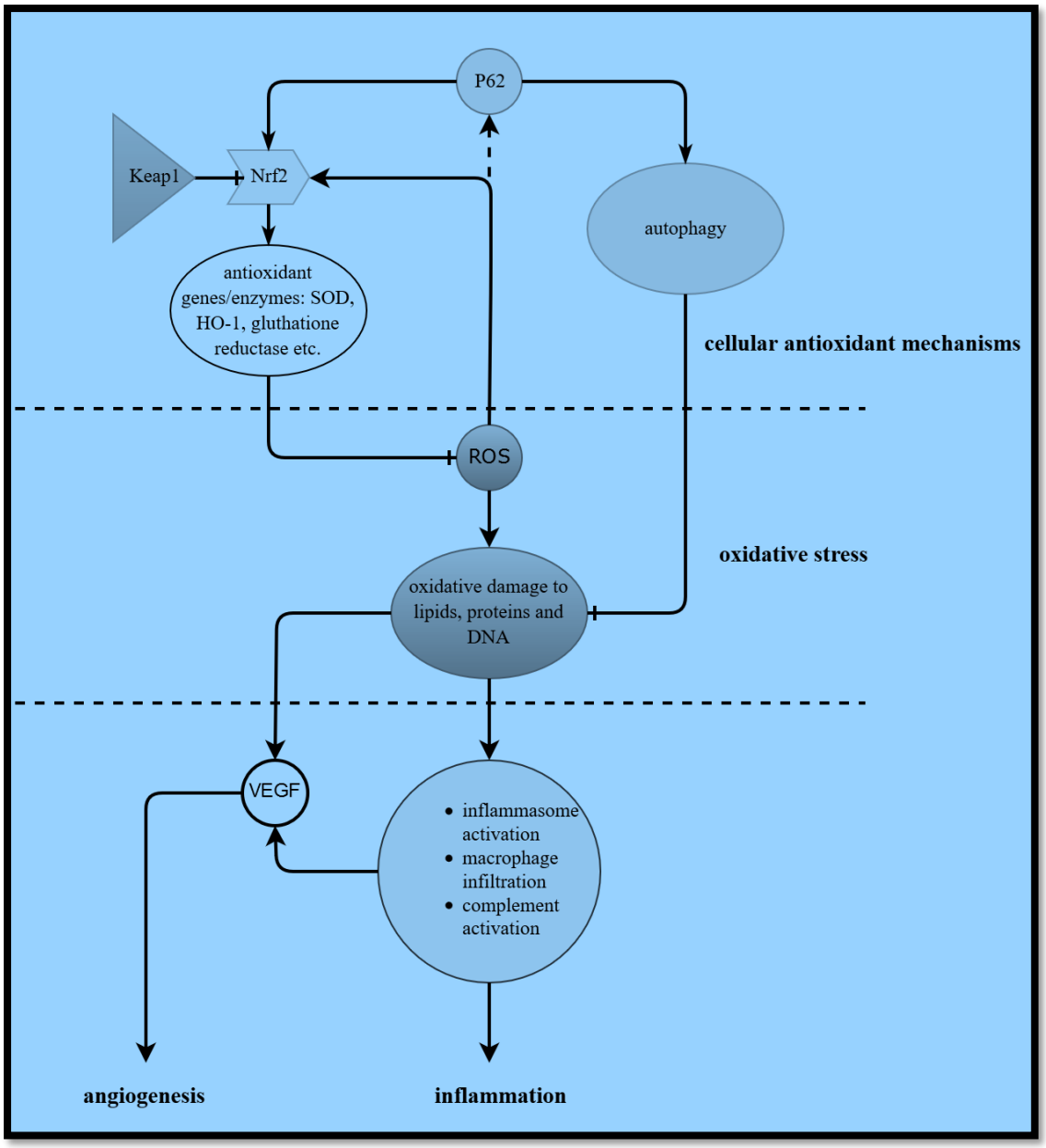


Figure 2. 2: The interrelationships between the molecular mechanisms involved in AMD show the potential therapeutic role of autophagy and NRF2 activation in the disease.

Oxidative damage to lipids, proteins, and DNA appears to be the primary insult leading to age-related macular degeneration. The accumulation of lipids due to an inhibition of lipid metabolism promotes oxidative stress by increasing lipid peroxidation in the retina. Oxidative stress could initiate inflammation through the activation of the inflammasome, complement and macrophages. Oxidative stress may also upregulate VEGF expression in the retina and induce choroidal neovascularization. The antioxidant mechanisms, including autophagy and NRF2, which are upregulated under oxidative stress counteract further oxidative damage and maintain retinal homeostasis.

CHAPTER III

Protective role of autophagy and NRF2 in hydroquinone-induced oxidative stress and death of human RPE cells

Introduction

Cigarette smoking is a major risk factor in age-related macular degeneration (AMD). According to the findings of an epidemiological study which was conducted among 7 countries in Europe, smokers were 4.8 times and 2.6 times at risk of developing dry AMD and wet AMD respectively compared to non-smokers (Chakravarthy et al., 2007). Smoking is however practiced by a significant proportion of adults worldwide (Myers et al., 2014). Extrapolating these results obtained from 187 countries to the world's population gives a total of about 967 million tobacco smokers, who are exposed to the increased risk of developing AMD (Ng et al., 2014).

Oxidative stress is one of the primary injuries induced by cigarette smoking on the human body including blood vessels, serum and blood cells, lungs, muscle tissue, human central nervous system as well as the gamete cells (Durazzo et al., 2016; Mons et al., 2016; Saleh et al., 2002). Partly, the propensity for cigarette smoking to induce oxidative stress is because of the rich composition of free radicals, and non-radical oxidants identified in cigarette smoke (Reilly et al., 2017). In fact, the plasma concentration of the smoke oxidant hydroquinone (HQ) is twice as higher in smokers compared to that of non-smokers (Deisinger et al., 1996). The hallmarks of oxidative stress are increased levels of lipid, protein and/or DNA damage (Vaváková et al., 2015). The oxidative damage to the biomolecules occurs due to the higher rate of reactive oxygen species (ROS) generation relative to the lower activities of antioxidant enzymes

such as SOD, CAT, and glutathione peroxidase (GSH-Px) in smokers (Chávez et al., 2007). Due to the widespread nature of the oxidative damage caused by cigarette smoking on the body, it has been implicated in neuronal cell death and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and AMD (G et al., 2017; S. S. Yu et al., 2016).

A huge body of evidence from human subjects with AMD and animal models of the disease supports the involvement of oxidative stress in AMD. Studies have reported increased lipid peroxidation, protein oxidation and DNA damage in the serum of AMD subjects and the retina from donors' eyes with AMD, compared to normal non-AMD cohorts (Crabb et al., 2002; Ethen et al., 2007; Renganathan et al., 2008; Totan et al., 2009). Also, in-vitro studies using cultured retinal pigment epithelial cells from AMD donors' eyes revealed higher ROS production and malfunctioning of the mitochondria (Golestaneh et al., 2017). Oxidative damage to mitochondrial and nuclear DNAs was observed in the retinal pigment epithelium (RPE) of AMD subjects (Blasiak et al., 2013; Terluk et al., 2015). In addition to the human AMD studies which show an association between oxidative stress and AMD, studies have also demonstrated a cause-effect relationship between oxidative stress and AMD in the wild-type C57 mice (Espinosa-Heidmann et al., 2006; Marin-Castaño et al., 2006; A L Wang et al., 2009). It was shown that chronic exposure of mice to cigarette smoke or HQ induced apoptosis in the RPE and photoreceptors, increased subretinal deposits, and thickening of the Bruch's membrane (Espinosa-Heidmann et al., 2006; Marin-Castaño et al., 2006; A L Wang et al., 2009). They reported that the RPE was the primary target of the oxidative injury before other signs of retinal degeneration were observed (Bertram, Baglole, Phipps, Libby, et al., 2009). On the other hand, human RPE cells have also been found to be

highly resistant to oxidative damage from various oxidants (Lili Lu et al., 2006). The reason why the RPE is the first site of oxidative damage in the development of AMD remains elusive. Therefore, identifying the molecular mechanisms underlying the oxidative damage of cigarette smoke would potentially reveal new therapeutic targets for the intervention of AMD.

Autophagy is the means by which cells recycled damaged molecules and obsolete organelles via lysosomal degradation (Giordano et al., 2013). The NRF2 pathway is particularly known for its regulation of antioxidant genes and enzymes involved in the detoxification of intracellular ROS (de Vries et al., 2008). These two cellular mechanisms apart from being functionally related in promoting cell survival are closely linked to each other by the protein called p62/ SQSTM1 (Jiang et al., 2015). During autophagy induction, p62 serves as a cargo receptor facilitating in lysosomal degradation of substrates (Lippai & Low, 2014). Also, p62 promotes the activation of the master antioxidant NRF2 transcription factor by disrupting the NRF2-Keap1 complex (Silva-Islas & Maldonado, 2018). Recently, autophagy and NRF2 pathways were discovered as essential cellular antioxidant defense mechanisms for cell survival under oxidative stress. The dysregulation of these mechanisms has been associated with increased susceptibility of neurodegenerative diseases including AMD (de Vries et al., 2008; Giordano et al., 2013; J. A. Johnson et al., 2008).

To explain the oxidative damage of the human RPE by cigarette smoke, we hypothesized that the smoke oxidant HQ may impair the essential cellular antioxidant pathways, leaving the human RPE vulnerable to oxidative damage. We, therefore, studied the toxicity of HQ on cultured human RPE cells compared to H_2O_2. When both oxidants generated a similar level of ROS, the cells were more susceptible to oxidative damage from HQ. Our further investigation to determine how each of the oxidants

affected the autophagy and NRF2 pathways showed that there were significant differences relating to the p62 and NRF2 protein expression. Whereas p62 and NRF2 were upregulated in the cells incubated with H_2O_2, both were downregulated at the transcription and protein level by HQ. It was also found that the changes in p62 and NRF2 by HQ were independent of autophagy or proteasome degradation. These suggest that HQ might directly impair the activation of the NRF2 antioxidant mechanism. These findings significantly advance our understanding of the association between cigarette smoking and AMD.

Methodology

Culturing of ARPE-19 cells

The human RPE cells (ARPE-19, ATCC® CRL2302 ™) were cultured with the commercially available Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen-Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin antibiotic mixture (Thermal Fisher Scientific, Rockford, IL, USA). Cells were incubated at 37ºC in a humidified atmosphere of 5% carbon dioxide. Cell medium was changed every 3 days until cells reached 90% confluency for use in experiments. Passages 16-25 were used for all experiments. According to the supplier (ATCC), the cells form stable monolayers, which exhibit morphological and functional polarity that could be carried for over 30 passages (Available:https://www.biocompare.com/20260-Miscellaneous-Human-Cell-Lines/875864-ARPE19/). Data also support that appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells (W. Samuel et al., 2017).

Cell viability assay and morphology

Briefly, ARPE-19 cells of seeding density $1x10^6$ cells/well was grown on a 6-well plate/35 mm MatTek glass-bottom dish (MatTek Corp., MA, USA) until ready for experiment. After 24 h starvation, cells were incubated with different concentrations of HQ (hydroquinone, Sigma-Aldrich, H9003) or H_2O_2 (Hydrogen peroxide 30%, 107209, Merck Millipore) in the presence of NAC (N-Acetyl-L-cysteine, A9165, Sigma-Aldrich) or without. Treatments were given in triplicate. Briefly, the Trypan blue dye exclusion assay protocol used in determining cell viability involved trypsinization of cells, followed by centrifugation at 1500 rpm for 5 min and staining with 0.4% trypan blue solution (Sigma-Aldrich, T6146). Then, the ratio of viable cells to nonviable cells was computed using a hematocytometer chamber under a microspcope.

An assessment of the morphology of the live cells grown in a 35 mm MatTek glass-bottom dish was performed using an inverted confocal microscopy (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam) and phase contrast with 20X magnification.

CM-H2DCFDA assay for intracellular ROS

Cells were plated onto 96-well plates at $5x10^4$ cells/well overnight. On the next day, the medium was discarded from cells, washed with PBS and incubated with 5 μM CM-H2DCFDA (C6827, Invitrogen) in the dark at 37°C for 1 h. Next, the ROS dye-imbibed cells were incubated with various concentrations of HQ or H_2O_2 for 1 h. The probe that was taken up into the cells was converted to dichloro dihydrofluorescein (DCFH) by intracellular ROS. Non-fluorescent DCFH was oxidized by the intracellular ROS to its

fluorescent form. Fluorescence intensity was then measured at 483 nm against 530 nm as reference using a Clariostar microplate reader (BMG Labtech, Offenburg, Germany).

Treatments and protein extraction

Serum-starved cells of 80-90 % confluency in 100 mm dish were incubated with various concentrations of HQ or H_2O_2 in the presence of CQ (Chloroquine diphosphate salt, C6628, Sigma-Aldrich), MG132 (M8699, Sigma-Aldrich) or vehicle. After treatment, cells were trypsinized, centrifuged, washed twice with PBS and stored at -80ºC for protein or RNA extraction. Proteins were extracted using ice-cold 1X RIPA lysis buffer [0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA (Millipore)] containing 1:100 protease inhibitor cocktail (Thermo Scientific). Samples were sonicated for 1 hour on ice at 4^0C, followed by centrifugation at 18000 x g for 30 mins at 4^0C. Supernatant was obtained, and protein concentrations were measured with BioRad Protein Assay (BioRad).

Protein carbonyl assay

Protein carbonyl concentration was measured in protein samples by spectrophotometry using the Oxiselect™ protein carbonyl ELISA kit (Cell Biolabs, STA-310) according to the manufacturer instructions. Briefly, the protein samples already prepared from cells treated with HQ, H_2O_2 or without were incubated with 1% streptomycin sulfate (Sigma-Aldrich, S9137) and diluted to the specified 10µg/ml protein concentration for the assay. Protein samples were then adsorbed onto a 96-well plate for 2 h at 37ºC and derivatized to DNP hydrazine. There was probing with anti-DNP antibody and followed by HRP conjugated secondary antibody. The absorbance of each well was measured at

450 nm wavelength using a microplate Reader plate (Ao, Azure Biosystems Inc., Dublin, USA).

Proteasome Activity Assay

Proteasome activity in cell samples was measured using a fluorogenic AMC-tagged substrate to detect chymotrypsin-like activity, following a protocol for the kit (Biovision proteasome activity assay kit, San Francisco, CA). Briefly, ARPE-19 cells were incubated with HQ or vehicle for 2 h, lysed with 25mM Tris-HCl buffer for 60 min at 4 °C, and the supernatant collected by centrifugation at 13,000 rpm for 10 min at 4 °C. Cell lysate of samples was loaded onto 96-well plate in duplicate for incubation with the fluorescent substrate at 37 °C for 30 min in the presence of MG132 (proteasome inhibitor) or without. The proteasome activity assay takes advantage of the chymotrypsin-like activity, utilizing an AMC-tagged peptide substrate which releases free, highly fluorescent AMC in the presence of proteolytic activity. Fluorescence intensity was then measured at 350 nm against 440 nm as reference using a Clariostar microplate reader (BMG Labtech, Offenburg, Germany). The protein concentration of samples was used for the normalization of data.

Immunoblotting for LC3-II, p62, and NRF2

An equal amount of protein (total protein of 30 µg) extracted from cell samples of different treatments was loaded onto wells of separating gel for SDS-PAGE electrophoresis (10% SDS-PAGE gels). All samples were denatured by the addition of β-Mercaptoethanol (M6250, Sigma-Aldrich) and incubation at 90^{o}C for 5 min. Proteins were electrotransferred from gel to an Immobilon-FL PVDF membrane (Millipore) for 2 h at 250 mA using cold pack and prechilled buffer to reduce the generation of heat.

After, the membrane was blocked using 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Bio-Rad Laboratories) for 1 hr at RT. Primary antibody incubation with anti-LC3 (NB100-2220, Novus Biologicals, dilution 1:1000), anti-P62 (2C11, Novus Biologicals, dilution1:2000) or anti-NRF2 (EP1808Y, Abcam, dilution 1:1000) was performed for 2 hr at RT. Next, the membrane was washed 3 times for 10 mins each time followed by incubation with horseradish peroxidase HRP-conjugated secondary antibodies including anti-mouse IgG (H+ L, A16066) and anti-rabbit IgG (H+L, A16110; Thermo Fisher Scientific, dilution 1:2000). Again, the membrane was washed 3 times, 10 mins each. ECL substrate solutions were mixed and incubated with the membrane for 5 mins, and immunoreactive bands were imaged using the Azure c600 imaging system. (Azure Biosystems; Dublin, CA). Quantification of bands was done using ImageJ analysis software. Protein expressions were normalized to B-actin (AC-15, Thermo Fisher Scientific dilution 1:2000).

shRNA knockdown of NFE2L2

Stable knockdown of *NFE2L2* in ARPE-19 cells was performed using lentivirus to deliver short hairpin RNA (shRNA). A density of 3 x 10^6 HEK293T cells was seeded in a 10 cm culture dish per dish. The cells were then transfected with lentiviral particles with either scrambled shRNA plasmid of shNFE2L2 shRNA, TRC numbers: TRCN TRCN0000007558 (Sigma Alrich) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Scramble shRNA-coding lentiviral vector was a gift from David Sabatini (Addgene plasmid # 1864). After 8 hours after transfection, the medium was removed and changed into a fresh medium for another 48 to 52 hours. Virions were collected and precipitated overnight using PEG before filtered using a 0.45μm filter and finally transduced into ARPE-19 cells for 48 hours. Afterward, cells

were then subjected to puromycin (1.0 μg/mL) selection for 10 days for the identification of resistant colonies.

Isolation of RNA, RT-PCR, and qPCR

Briefly, RNA extraction was performed using Trizol (Life Technologies) and following the manufacturer's protocol. RNA samples were free from contaminants; both the A260/280 and A260/230 ratios fell within 1.9 to 2.1 (Thermo Scientific™ NanoDrop). Next, cDNA was transcribed from 1 μg DNA-free RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and stored for conventional reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative PCR (qPCR).

For quantitative PCR, a PCR of reaction volume 10 μl per well was set up in triplicate using 2 μl cDNA template, 5 μl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), 1 μl nuclease-free water, and 1 μl of gene-specific primers for NRF2, P62, and B-Actin. Quantitative RT–PCR was run on a LightCycler 480 System II (Roche Diagnostics). The reaction mixture was incubated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. All qRT–PCRs were run using the LightCycler 480 System II (Roche Diagnostics). Fold changes were calculated using the change in the cycle threshold (ΔΔCT) method.

For the conventional RT-PCR, PCR reaction volume of 20 μl was carried out using the following: 1.5μl cDNA template, 10μl 2x Taq HS mix (R028A, Premix Taq™ DNA Polymerase Hot-Start Version), 1 μl each of forward and reverse primers (10 μM) and 6.5 μl nuclease-free water. Amplification was performed for 25 cycles at a denaturing temperature of 94℃ for 30 s, annealing temperature of 60 °C for 30 s and an extension

temperature of 72℃ for 30 s. The amplified products 10μl PCR was mixed with 2µl 6× DNA loading buffer and DNA signal analyzed by 1.5% agarose gel electrophoresis containing GelGreen nucleic acid stain (Biotium Inc., Hayward, CA, USA) and visualized under UV light (Gel Doc/ChemiDoc Imager, Azure, Dublin, CA, USA). B-actin was the reference gene used for normalization of expression of other genes.

Statistical analysis

GraphPad Prism and Excel were used for analyzing data and drawing of graphs. All data are presented as mean ± SD. For analysis to determine the difference between treatments, the unpaired t-test was used when two treatment groups were involved, and when three or more treatment groups were concerned the one-way ANOVA followed by Dunnett's multiple comparison tests was performed. Statistical significance was set at $p < 0.05$.

Results

Vulnerability of human RPE cells to the smoke oxidant HQ

Studies of the molecular mechanisms behind the increased risk of AMD among smokers have concluded that oxidative damage to the RPE was crucial, but the mechanism behind it is poorly understood (Bertram, Baglole, Phipps, Libby, et al., 2009; M Fujihara et al., 2008). However, it was shown in vitro that the human RPE cells were resistant to oxidative stress from different oxidants including H_2O_2 (Lili Lu et al., 2006). We, therefore, sought to determine whether the human RPE cells are vulnerable to HQ-induced oxidative stress relative to H_2O_2 –induced oxidative stress (Gough & Cotter, 2011). Hydroquinone (HQ) is an important cigarette smoke oxidant shown to induce RPE oxidative damage, dysregulate inflammatory cytokines and

vascular endothelial growth factor homeostasis and cause AMD-like retinal changes (Cao et al., 2013; Espinosa-Heidmann et al., 2006). Except for HQ, none of the other cigarette smoke oxidants including nicotine implicated in RPE damage has been shown to induce retinal degeneration *in vivo*. We studied the cytotoxicity of HQ and H_2O_2 on the ARPE-19 cells using the oxidative stress indicator CM-H2DCFDA, Trypan blue cell viability assay, and protein carbonyl assay. The human RPE cells incubated with various concentrations of HQ (0-100 µM) or H_2O_2 (0-1000 µM) for 2 h showed significant loss of cell viability dose-dependently (Fig. 3.1 A & B). The LC50 for HQ and H_2O_2 was about 50 µM and 700 µM, respectively. Also, both oxidants increased ROS (Fig. 3.1 A-D) and protein carbonyl dose-dependently (Fig. 3.1 C & D). Protein carbonyl is a reliable indicator of oxidative damage (Weber, Davies & Grune 2015). It was found that with similar levels of ROS induction in cells by the oxidants, the HQ treatment, however, had higher protein carbonyl level and lower viability compared to H_2O_2., For instance, 50 µM HQ induced ROS levels similar to 250 µM H_2O_2, but 50 µM HQ caused greater loss of viability (one-way ANOVA, $p < 0.001$; Fig. 3.1 A & B) and oxidative damage (one-way ANOVA, $p < 0.001$; Fig. 3.1 C & D) relative to the control. These results together support that the human RPE cells were more vulnerable to oxidative damage from HQ.

Oxidative stress mediates HQ-induced cytotoxicity

We studied the importance of oxidative stress in mediating HQ-induced cytotoxicity on the human RPE cells. N-acetyl cysteine (NAC) is an antioxidant known for its protection against oxidative stress in the retina through the enhancement of intracellular GSH (Atkuri et al., 2007; Palmero et al., 2001). Human RPE cells were incubated with or without NAC, followed by incubation with HQ. Compared to the cells exposed to

HQ alone, cells preincubated with NAC prior to their exposure to 50 µM HQ showed significantly higher cell viability which was dose-dependent (Fig. 3.2 A). Also, preincubation with the 0.5 mM NAC completely prevented the increase in protein carbonyl and reduction of cell viability by HQ (Fig. 3.2 A & B), thus showing that HQ-induced cytotoxicity in human RPE cells is mediated by oxidative stress.

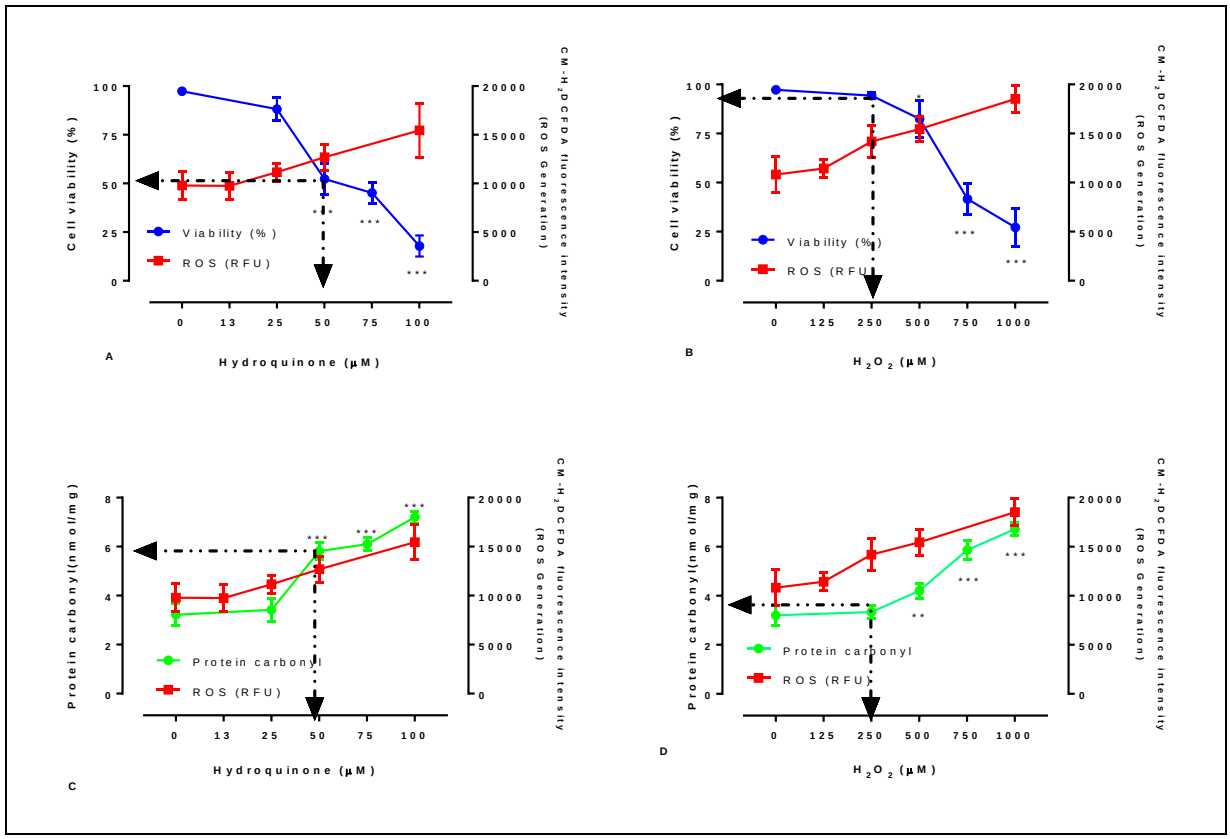


Figure 3. 1: ARPE-19 cells are more vulnerable to oxidative damage from hydroquinone (HQ) compared to H2O2.

(A-D) At similar reactive oxygen species (ROS) induction levels by oxidants, (A & B) the viability was lower and the (C & D) protein carbonyl level was higher for the HQ-treated-cells than H_2O_2-treated cells. Cells were incubated with various concentrations of HQ or H_2O_2 for 2 h. (A-D) CM-H2DCFDA assay was performed for ROS level in cells and fluorescence measured at λex: 483 nm and λem: 530 nm. (A & B) Cell viability was assessed by the trypan blue assay. (C & D) Protein carbonyl level was measured in whole-cell lysates using a kit (Cell Biolabs, STA-310). (A-D) Data represent the mean (± SD) of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

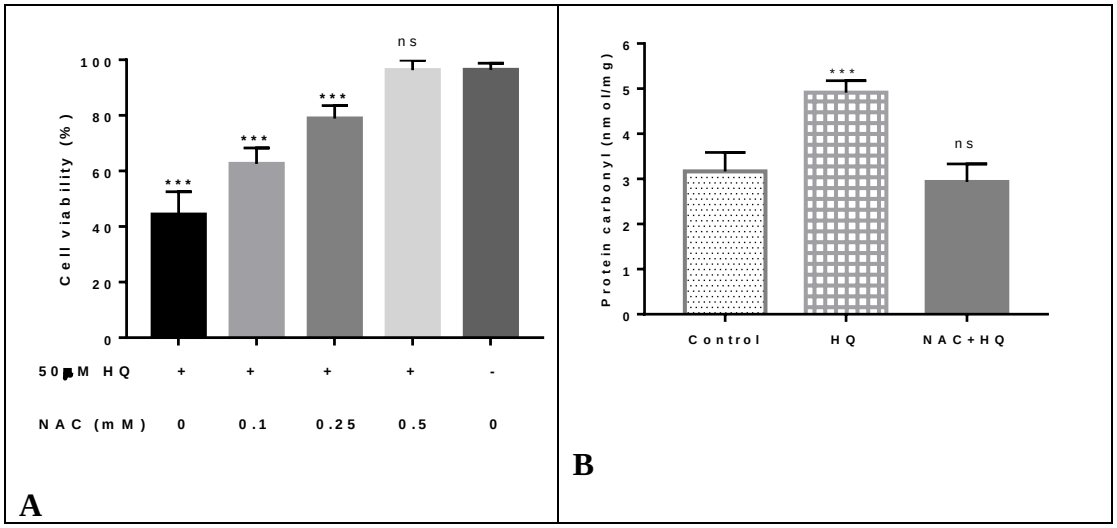


Figure 3. 2: Hydroquinone (HQ)-induced toxicity in ARPE-19 cells is mediated by oxidative stress.

(A & B) N-acetyl cysteine (NAC) protects against HQ. Cells incubated with NAC for 2 h, followed by incubation with 50μM HQ for 2 h showed improved cell viability and (B) lower protein carbonyl level. Cell viability was assessed by the trypan blue assay. Protein carbonyl level was measured in whole-cell lysates using Oxiselect™ protein carbonyl ELISA kit (Cell Biolabs, STA-310). Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. ***$p < 0.001$ vs control.

HQ impairs p62 and NRF2 at transcription and protein levels

Oxidative damage occurs due to an imbalance between ROS production and the antioxidant defense mechanisms in favor of the ROS (Sies, 1997). Autophagy and NRF2 activations protect against oxidative stress and neurodegeneration (de Vries et al., 2008). NRF2 is a master antioxidant transcription factor responsible for the upregulation of genes and antioxidant enzymes involved in neutralizing intracellular ROS. Also, the lysosomal degradation of oxidized proteins, lipids, and organelles helps in the recycling of biomolecules, preventing oxidative stress. We, therefore, argued that in addition to ROS production, HQ might compromise autophagy or NRF2 activation, accounting for the increased vulnerability towards oxidative damage. As 50 μM HQ or 250 μM H_2O_2 induced similar levels of ROS in the cells (Fig. 3.1 C & D), we compared their effects on protein expression levels of NRF2, LC3-II and the p62 molecule linking

both pathways (Jiang et al., 2015). The western blot results showed that H_2O_2 treatment was associated with an upregulation of LC3-II, p62, and NRF2 protein levels (Fig. 3.3 A-D). However, HQ treatment increased LC3-II but significantly decreased p62 and NRF2 (Fig. 3.3 A-D), supporting an inhibition of the p62/NRF2 antioxidant pathway in cultured human RPE cells. We performed qPCR to determine transcriptional changes in p62 and NRF2 mRNA levels by HQ or H_2O_2. Cells exposed to HQ showed significant downregulation of mRNA expressions for both p62 (0.5-fold, $p < 0.05$) and NRF2 (0.4-fold, $p < 0.001$), but upregulated by H_2O_2 exposure (p62: 1.8-fold, $p < 0.01$; NRF2: 1.42-fold, $p < 0.05$) compared to control (Fig. 3.3 E & F).

Furthermore, we studied the dose-response of HQ and H_2O_2 on p62 and NRF2 protein levels following 2 h of incubation. Similar to our earlier results, it was found that while HQ dose-dependently depleted p62 and NRF2 (left panel, Fig. 3.4 A, C & E), H_2O_2 upregulated both p62 and NRF2 (right panel, Fig. 3.4 B, D &F). Both oxidants, however, increased the LC3-II dose-dependently (Fig. 3.4 G & H). Collectively, these findings associate the inhibition of p62 and NRF2 with oxidative damage by HQ in human RPE cells.

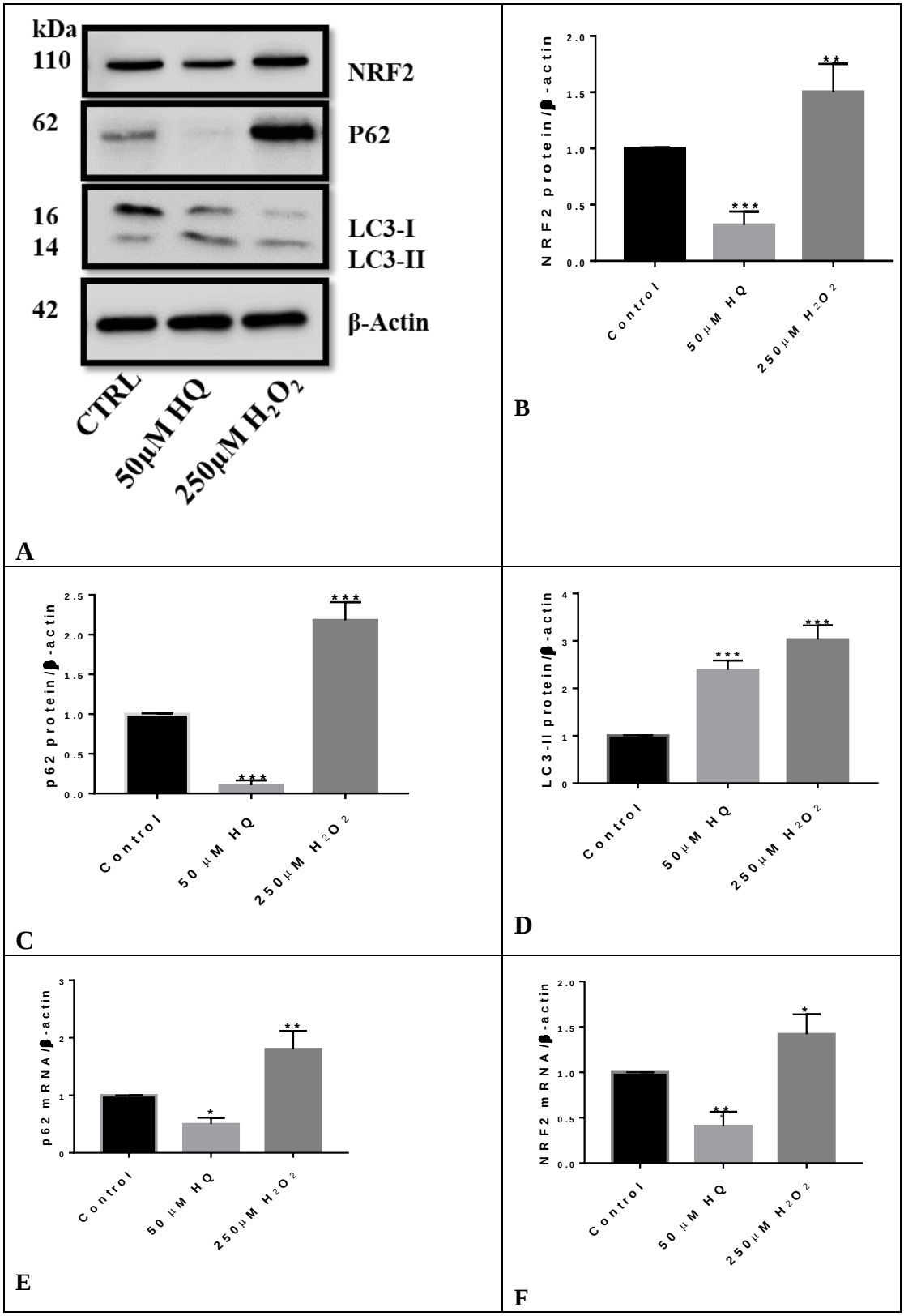
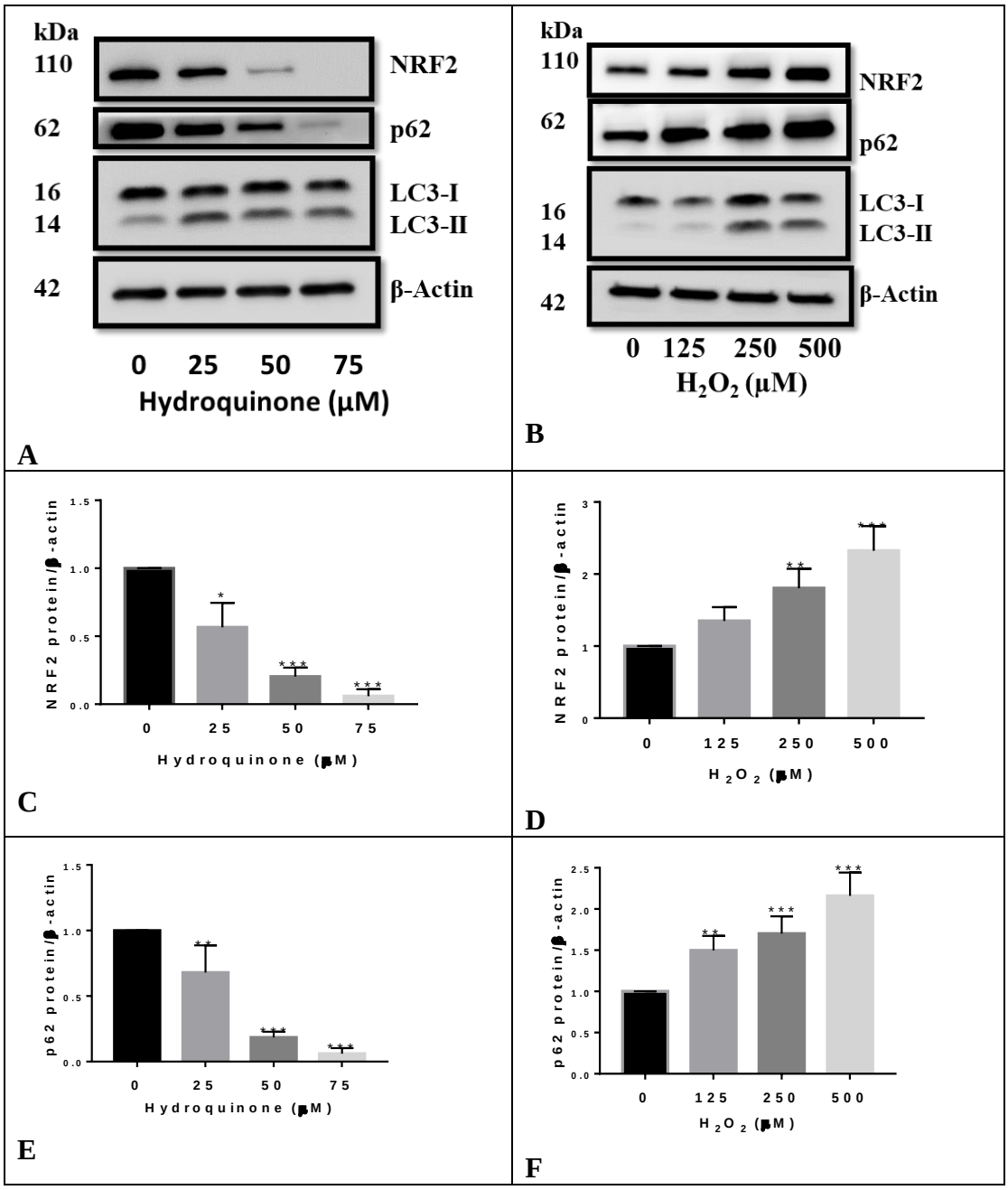


Figure 3. 3: Hydroquinone (HQ) impairs p62 and NRF2 at transcription and protein levels.

(A-F) Differential effects of HQ or H_2O_2 on LC3-II, p62 and NRF2 mRNA and protein expressions. ARPE-19 cells were incubated with 50 µM HQ, 250 µM H_2O_2 or vehicle for 2 h. (A) whole-cell lysate preparation for SDS-PAGE and immunoblotting with

appropriate antibodies. (B-D) Densitometry quantification of protein levels normalized to β-actin and expressed as a ratio of the control (vehicle). RNA extraction and RT-qPCR. The mRNA expression levels of p62 and NRF2 were normalized to B-actin and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.



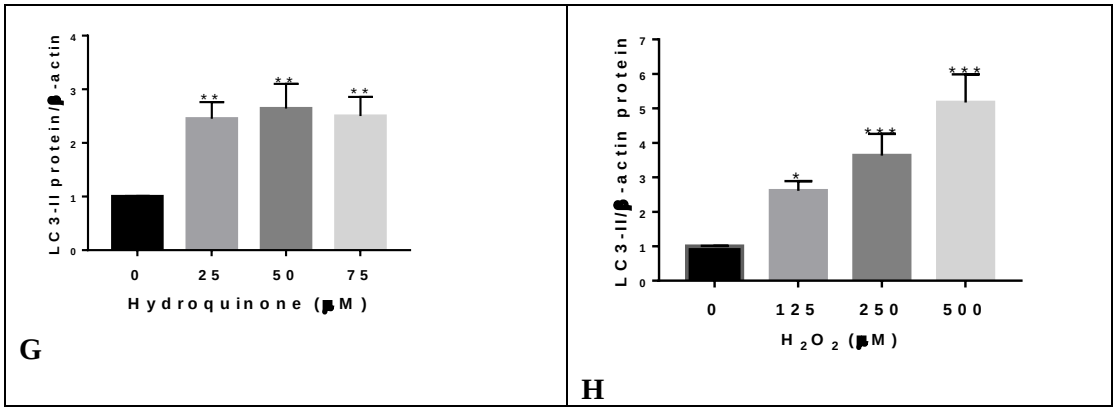


Figure 3. 4: Dose-response of hydroquinone (HQ) or H_2O_2 for p62, NRF2, and LC3-II protein expressions.

(A & B) Human RPE cells were incubated with various doses of HQ or H_2O_2 or vehicle for 2 h, and whole-cell lysate prepared for SDS-PAGE and immunoblotting with appropriate antibodies. (C-H) Densitometry quantification of protein levels normalized to β-actin and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

HQ's downregulation of p62 and NRF2 was rapid and sustained

It is known that NRF2 activates in response to oxidative stress (Nguyen et al., 2009). Hence, we investigated the temporal course of effects of HQ and H_2O_2 on the p62 and NRF2 protein expressions and determined whether it varied with time. RPE cells were incubated with 50 µM HQ, 250 µM H_2O_2 or vehicle for 0.5 h, 2 h, 4 h, or 24 h, and protein level analyzed by western blot. A significant and rapid decline in the p62 and NRF2 protein expressions was observed up to the 4 h in the HQ-treated cells compared to control, becoming overexpressed only at the 24 h (Fig. 3.5 A, C & E).

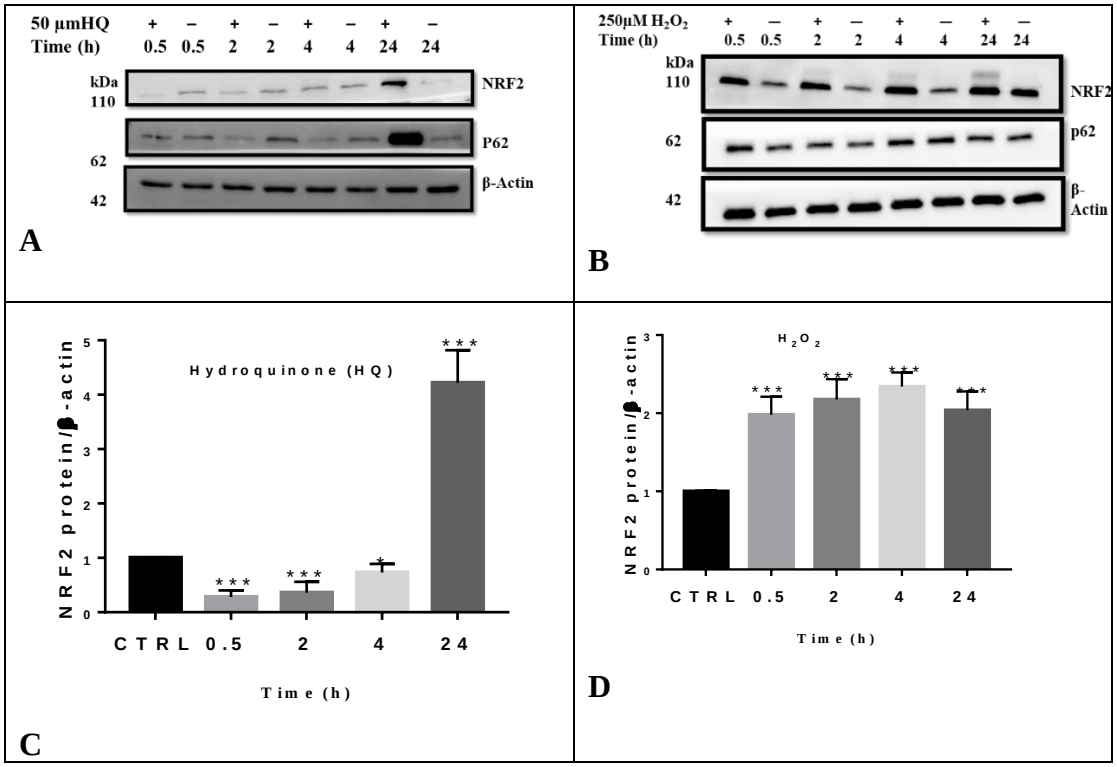
On the contrary, H_2O_2 treatment rapidly showed increased p62 and NRF2 protein expressions throughout all time points compared to control (Fig. 3.5 B, D & F). Even though our results may support the activation of the p62/NRF2 antioxidant pathway under oxidative stress, it was obvious that HQ did not only delay the activation but also inhibited it for prolonged hours contributing to oxidative damage by the generated ROS.

Inhibition of p62/NRF2 is independent of autophagy or proteasome degradation

Proteolytic degradation of p62 and NRF2 in cells takes place in the lysosome and proteasome, respectively (Jaakkola & Pursiheimo, 2009; D. Stewart et al., 2003). Hence, a decline in the p62 or NRF2 proteins could be because of increased lysosomal degradation (i.e., autophagy) or proteasomal degradation. Therefore, we investigated whether upregulated autophagy or proteasomal degradation underlies the changes in p62 or NRF2 a protein's expression levels by inhibition of proteolysis, which should result in the accumulation of proteins. To test whether the change in p62 protein levels was mediated by autophagy, a lysosomotropic drug (chloroquine (CQ)) was used to inhibit lysosome degradation in cells (Mauthe et al., 2018), followed by examining the protein expressions of the autophagosome marker LC3-II and autophagy substrate p62. Cells were incubated with 50 μM CQ for 8 h followed by incubation with HQ or vehicle for 2 h. The dose of CQ and its duration of incubation were chosen as it provided optimal autophagy inhibition with minimal toxicity according to a previous study (P. M. Chen et al., 2011). Compared to control, CQ alone significantly increased the LC3-II and p62 in the cells, due to block of the autophagy flux (Mauthe et al., 2018). In the presence of CQ, however, HQ increased LC3-II but reduced p62 (Fig. 3.6 A, C & E). An increase in LC3-II by HQ in the presence of maximal autophagy inhibition by CQ supports an increase in autophagy flux by HQ. But, since p62 was decreased by HQ in the presence of CQ which impaired the autophagy flux, it is evident that autophagy is not the main mechanism by which HQ depletes p62.

Also, there was an assessment of the proteasome activity level to find out the involvement of proteasome degradation in the HQ-induced NRF2 changes. Data showed that the proteasome activity was increased by HQ dose-dependently (Fig. 3.6

B), suggesting increased proteasome degradation contributed to some extent in the depletion of NRF2 at the protein level. As a result, we studied changes in NRF2 protein expression by HQ, in the presence of MG 132, a potent proteasome inhibitor. The results showed that cells incubated with MG132 increased NRF2 expression compared to control, as was expected (1.26-fold, p = 0.02, Fig. 3.6 D & F). However, incubation of cells with HQ in the presence of MG132 still showed a decline in NRF2 protein expression compared to control (0.64-fold, p = 0.002, Fig. 3.6 D & F), suggesting also that the mechanism by which HQ downregulated NRF2 might be independent of proteasomal degradation.



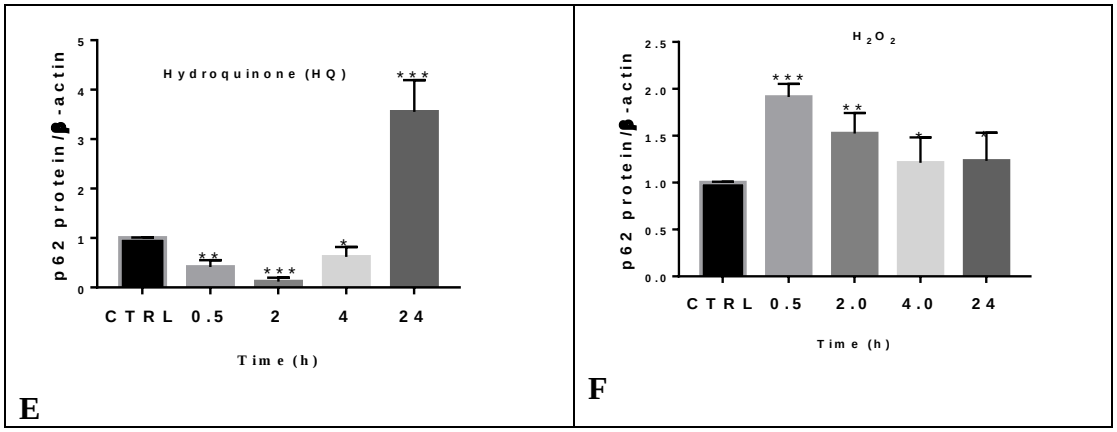
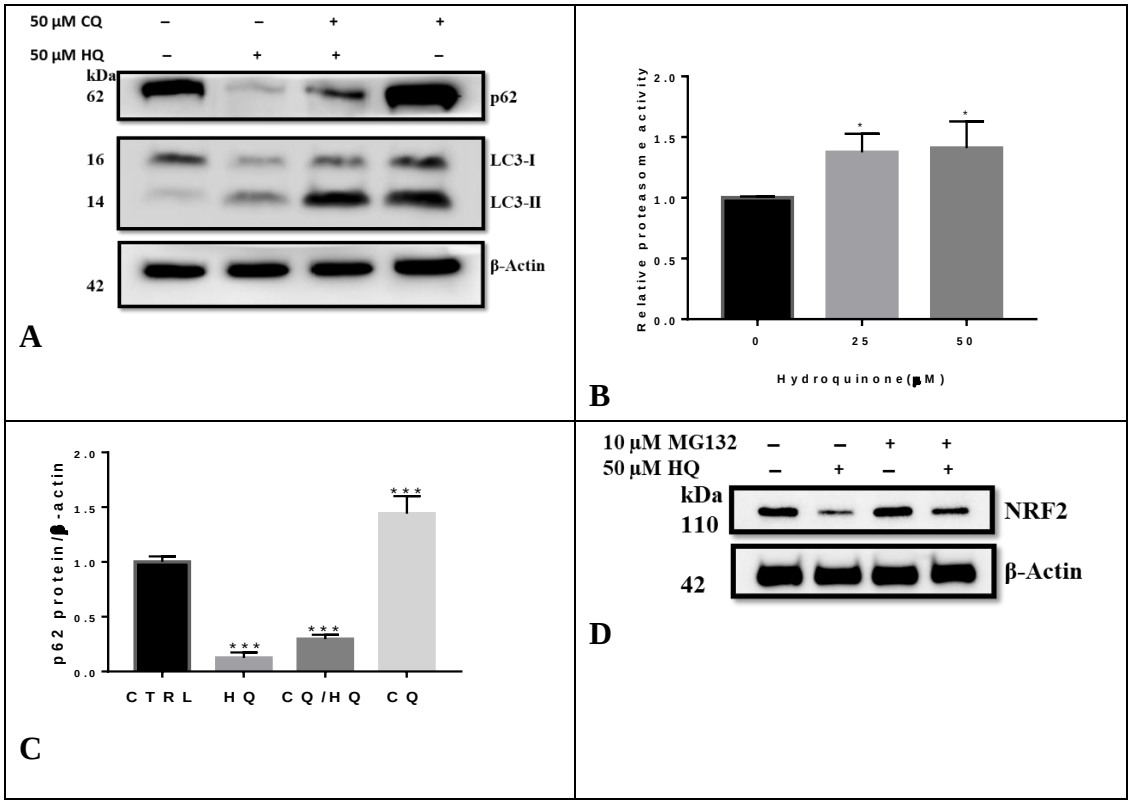


Figure 3. 5: Effect of hydroquinone (HQ, left panel) or H_2O_2 (right panel) on p62 and NRF2 protein expressions over time.

(A & B) p62 and NRF2 expressions were (A) rapidly downregulated by HQ but (B) remain upregulated by H_2O_2 p62 and NRF2 at all the time points. Cells were incubated with 50 µM HQ, 250 µM H_2O_2 or vehicle for different time points, and whole-cell lysate prepared for SDS-PAGE and immunoblotting with appropriate antibodies. (C-F) Densitometry quantification of protein levels normalized to β-actin and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using a one-way ANOVA followed by the Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.



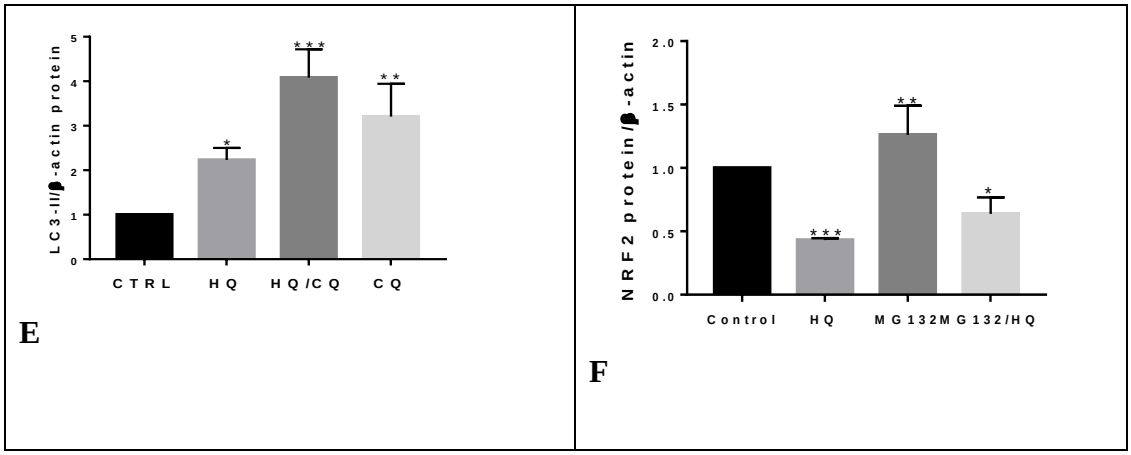


Figure 3. 6: Hydroquinone-induced decline in p62 and NRF2 proteins is independent of autophagy or proteasome degradation.

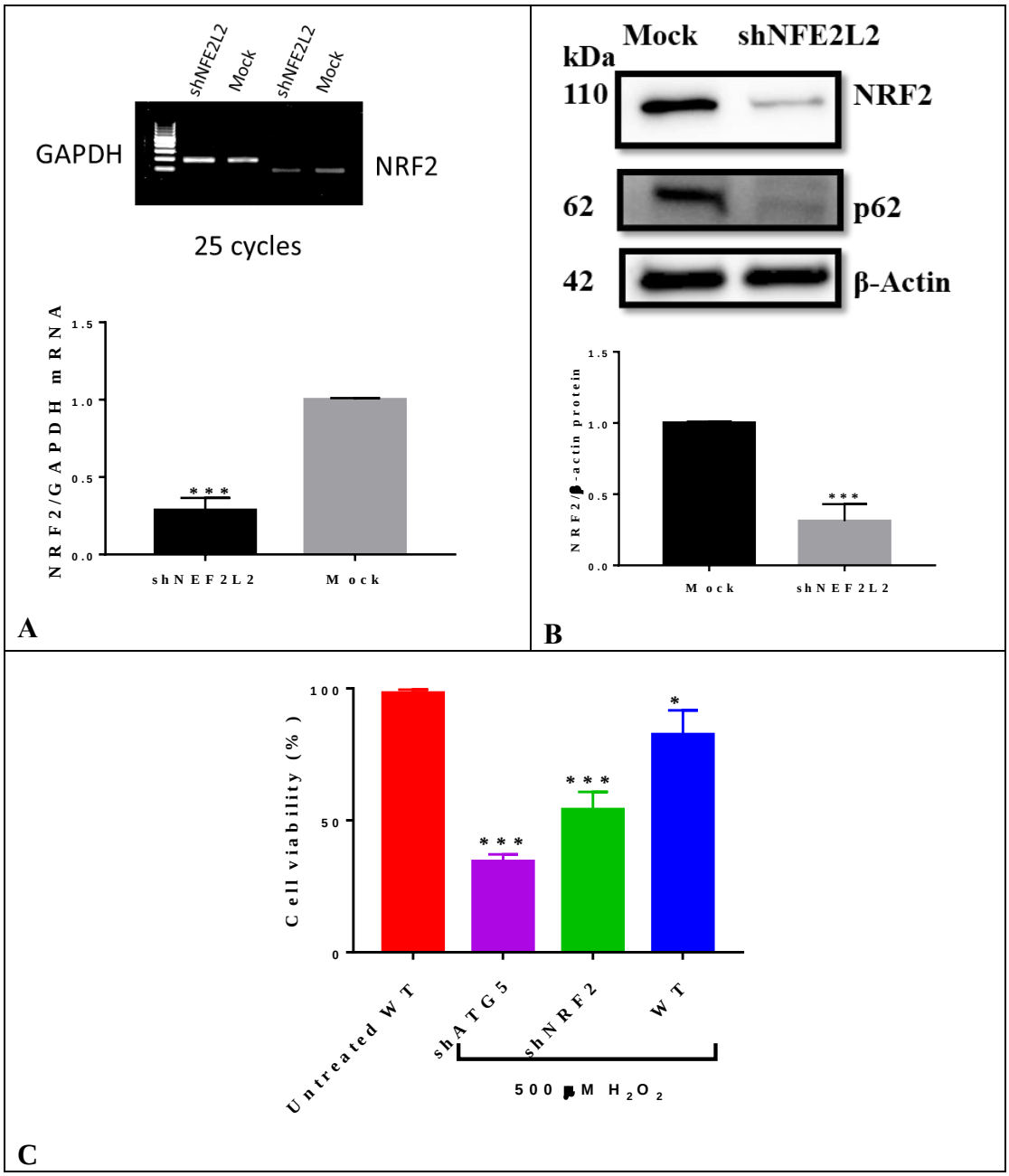
(A-F) HQ inhibits p62 and NRF2 expression in cells with impaired autophagy or proteasome degradation. ARPE-19 cells were incubated with (A) chloroquine (CQ) or (D) MG132 or for 8 h, prior to incubation with HQ for 2 h. Whole-cell lysate was prepared for immunoblotting with the requisite antibodies. (C, E, F) Densitometry analysis of protein levels normalized to β-actin and expressed as a ratio of the control. (B) Proteasome level was measured (Biovision proteasome activity assay kit) in lysates from cells treated with varying doses of HQ. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

Inhibition of p62/NRF2 renders RPE cells vulnerable to H_2O_2

To demonstrate that NRF2 inhibition contributes to oxidative stress in human RPE cells, there was knockdown of the NRF2 gene in RPE cells by transfection with *NFE2L2* shRNA for 48 h and assessment of the changes in the viability of cells upon exposure to H_2O_2. According to PCR and western blot results (Fig. 3.7 A & B), NRF2 and p62 were downregulated in the shNFE2L2 cells compared to the mock (scrambled RNA transfected cells). As expected, incubation of shNFE2L2 cells with 500 µM H_2O_2 for 2 h showed a greater loss in viability compared to the mock or wild-type cells (Fig. 3.7 C).

In addition, we thought that provided p62/NRF2 depletion was crucial in oxidative stress-induced death of the RPE cells, then lethal doses of H_2O_2 might as well inhibit expression of these two proteins. Investigating the dose-response changes in p62 and

NRF2 protein expressions by H_2O_2 in the wild-type human RPE cell, we found that indeed lethal doses of H_2O_2 impaired p62 and NRF2 protein levels (Fig. 3.7 D). Thus, H_2O_2 has a biphasic dose-response effect on both p62 and NRF2 protein levels, upregulated by lower doses and downregulated by high doses of H_2O_2. These findings strongly support that the downregulation of p62/ NRF2 in human RPE cells increased their susceptibility to oxidative damage.



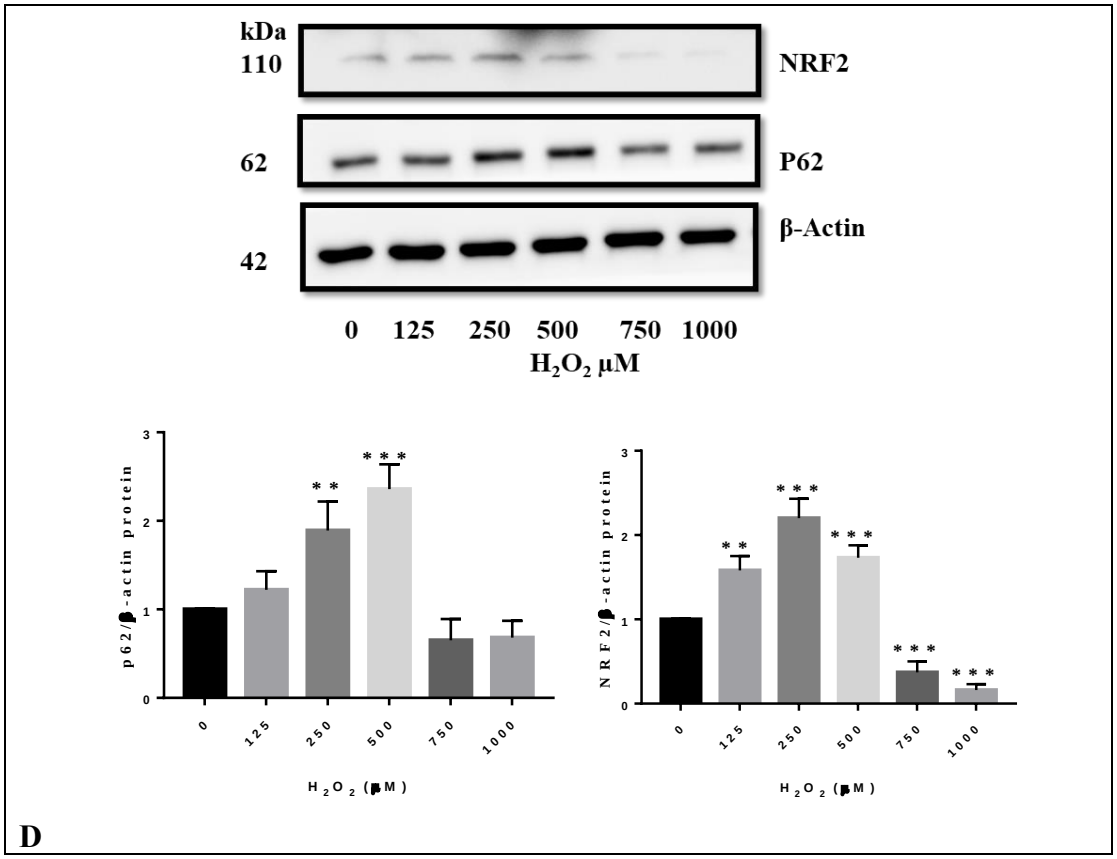


Figure 3. 7: Inhibition of p62/NRF2 renders RPE cells vulnerable to H_2O_2.

(A-C) *NFE2L2* shRNA transfection impairs NRF2 and p62 protein levels in ARPE-19 cells, rendering the shNFE2L2 cells susceptible to H_2O_2. (D) Lethal doses of H_2O_2 impairs p62 and NRF2 protein levels in wild-type ARPE-19 cells. Wild-type cells or cells transfected with scrambled or *NFE2L2* shRNA for 48 h were incubated with doses of H_2O_2 or vehicle for 2 h. (A) RNA extraction from cells and quantification of NRF2 mRNA expression level by RT- PCR. (B & D) Immunoblotting with requisite antibodies for p62 and NRF2 protein levels using whole-cell lysate. (C) Trypan blue assay was performed to determine the dose-response of H_2O_2 for the viability of cells. (A, B & D) Densitometry quantification of mRNA and protein expression levels normalized to β-actin and expressed as a ratio of the control. Statistical analysis was performed using unpaired *t-test* or one-way ANOVA followed by Dunnett's multiple comparison *post hoc* tests. Non-significant (ns), ***$p < 0.001$ vs control.

Discussion

While evidence supports the involvement of multiple molecular pathways in AMD, including inflammation, angiogenesis and cellular metabolism, oxidative damage to the RPE appears to be crucial. For instance, subjecting the RPE to oxidative stress could lead to NLRP3 inflammasome activation and promote inflammation in the retina (Cruz-

Guilloty et al., 2014; Kauppinen et al., 2012). Also, oxidative stress promotes has been linked to increased VEGF expression in the retinal pigment epithelium (Byeon et al., 2010), a necessary factor in the inducement of choroidal neovascularization in wet AMD. In addition, evidence from studies in human subjects and laboratory animals have corroborated that cigarette smoke or its prooxidant hydroquinone is a major risk factor in AMD (Chakravarthy et al., 2007; M Fujihara et al., 2008; Khan et al., 2006; A L Wang et al., 2009). All this information together underscores the central role of oxidative damage to the RPE in the development of AMD. Therefore, studying the mechanisms involved in hydroquinone-induced RPE oxidative damage might be beneficial for understanding the early events in AMD (Deisinger et al., 1996; Ong et al., 1994). In this study, we showed that doses of HQ generating reactive oxygen species (ROS) of similar levels relative to H_2O_2 induced greater oxidative damage and cell death, indicative of increased vulnerability to HQ. And, this vulnerability to HQ was linked to the downregulation of important proteins, p62 and NRF2 (Fig. 3.8), which modulate the cellular antioxidant defense (Katsuragi et al., 2016; Silva-Islas & Maldonado, 2018). These findings explain why cigarette smoking might be so harmful to the RPE than other potential inducers of oxidative stress in the retina.

Oxidative stress mediates HQ-induced cytotoxicity

Hydroquinone has been reported to cause oxidative damage to diverse cells of the human body, including cells of the lungs, liver, and eye (C. Chen et al., 2014; Luo et al., 2008; Peng et al., 2013). This study corroborates the findings of previous studies by showing that HQ generates ROS accumulation dose-dependently, increased protein carbonyls and increased loss of viability in the human RPE cells. In addition, it was demonstrated that pretreatment of the cells with NAC, a potent inducer of GSH synthesis, protected against the HQ-toxicity, to validate the central role of oxidative

stress. Besides oxidative stress, others have also reported that HQ dysregulated phagocytosis, inflammatory cytokines, angiogenic factors, and induced actin rearrangement in the human RPE cells (Cao et al., 2013; Pons & Marin-Castaño, 2011). We speculate that some of these other effects of HQ may be secondary to oxidative damage based on the observation that cells were entirely protected from the HQ-induced toxicity in the presence of the potent antioxidant precursor NAC. However, we caution against extrapolating this finding to all human cells or beyond the cellular level as the response of other cells may be unique, even though we are highly confident of a similar response.

Molecular mechanism involved in the increased vulnerability to HQ

Results from our study showed that autophagy and NRF2 pathways were affected by oxidative stress. Their temporal effects on the p62/NRF2 pathway were however different. HQ rapidly downregulated the expression of p62 and NRF2 protein levels until the 24 h, contrary to oxidative stress by H_2O_2 of similar ROS generation levels. The recovery of cells at the 24 h was perhaps related to the stability of HQ in the culture medium which may be affected by the metabolism of the RPE cells or external factors including hydrolysis, oxidation, photolytic and thermal conditions (Borges et al., 2018). The NRF2 is a ubiquitously expressed transcription factor in cells known for the activation of antioxidant response element (ARE) responsible for regulating the expression of the antioxidant enzymes (Nguyen, Nioi, & Pickett, 2009). Thus, activation of NRF2 under conditions of oxidative stress enhances the expression of antioxidant genes and enzymes, including GSH-Px, catalase (CAT) and superoxide dismutase (SOD) or protection against oxidative stress (Finkel et al., 2000). An increase in NRF2 expression is, however, necessary for its activation and translocation into the

nucleus where the induction of the antioxidant enzymes finally occurs. The p62 protein molecule plays a pivotal role in both NRF2 and autophagy activation (Jiang et al., 2015). In autophagy, p62 acts as an adaptor binding ubiquitylated protein aggregates for delivery to the autophagosomes, and subsequently for lysosomal degradation. Also, p62 is involved in the upregulation of the NRF2 expression through its interaction and sequestration of Keap1, preventing the ubiquitylation and proteasomal degradation of the NRF2-Keap1 complex (Komatsu et al., 2010). The upregulation of NRF2 expression by p62 referred to as the noncanonical pathway of NRF2 activation, is favored when there is an accumulation of p62 (Komatsu et al., 2010). The downregulation of p62 and NRF2 expressions, therefore, implies an inactivation of the NRF2 antioxidant pathway, which may explain the susceptible to HQ-related oxidative damage. Thus, HQ might pose a unique 'oxidative challenge' compared to the oxidative stress from other oxidants, as the increase in ROS occurs in an environment of impaired oxidant defense in the RPE cells (Fig. 3. 8). In a cell, the metabolic process involving mitochondria and peroxisomes, as well as diverse cytosolic NADPH oxidases (superoxide-generating system) may generate ROS in the RPE. In addition, external agents including UV, ionizing radiation, nutrition, and environmental toxins can also trigger ROS production, making the NRF2 antioxidant pathway essential for survival (Finkel & Holbrook, 2000). By further demonstrating that 1) NRF2 inhibition by knockdown of NFE2L2 increased the toxicity of H_2O_2 in the RPE cells and 2) lethal doses of H_2O_2 showed similar inhibitory effects on p62 and NRF2 emphasizing the significance of the p62/NRF2 pathway in oxidative stress.

The involvement of autophagy in the HQ-induced oxidative damage might be expected as other conditions of oxidative stress have been found to activate autophagy (Y. Chen et al., 2009). The upregulation of autophagy by both HQ and H_2O_2 in the RPE cells

may suggest that the autophagy mechanism is a more versatile antioxidant mechanism, as it remained functional under the two conditions of oxidative stress. Autophagy might not directly neutralize intracellular ROS, but it could inhibit oxidative damage by eliminating the damaged mitochondria contributing to increased ROS levels through a process referred to as mitophagy (Ciccarone et al., 2019). Also, the activation of autophagy was reported to facilitate the clearance of oxidized biomolecules such as lipids, proteins, and DNA (Baek et al., 2017; Mitter et al., 2014). This role of autophagy is believed to have an antioxidant effect and promote the survival of the RPE against oxidative stress (Mitter et al., 2014). Thus, autophagy could be a primary antioxidant mechanism for cells to depend on for survival under oxidative stress by HQ.

Our study found that the downregulation of these proteins was not related to changes in autophagy or proteasomal activation, which are known to be the main degradative pathways involved in the regulation of these proteins. Rather, we observed that the changes in p62 and NRF2 were associated with a decline in their gene expression levels, suggesting that the synthesis of these proteins might be impaired by HQ. In addition to the transcriptional changes in NRF2, it is also possible that the reduction in the p62 protein expression also contributed to the downregulation of NRF2 expression, since p62 is involved in the stabilization of NRF2 (Komatsu et al., 2010).

Conclusion

Our study implicates the dysregulation of autophagy and the NRF2/p62 pathway in the oxidative damage of human RPE cells. The inhibition of NRF2/p62 particularly underlies the increased risk of oxidative stress associated with the cigarette smoke oxidant HQ, advancing our understanding of the association between cigarette smoking and AMD. Thus, the targeting of these cellular and molecular pathways might be of therapeutic relevance in the management of AMD.

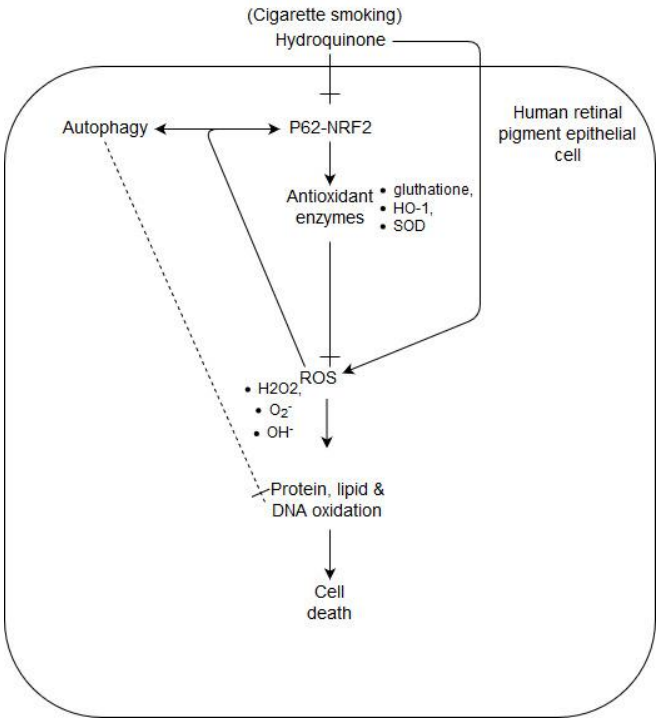


Figure 3. 8: A graphical abstract showing the mechanism behind the increased vulnerability to oxidative stress from hydroquinone.

Unlike oxidative stress due to H_2O_2, the cigarette-smoke oxidant rapidly impairs the NRF2 master antioxidant transcription factor and p62 inducing oxidative damage to the retinal pigment epithelium.

CHAPTER IV

Vitamins C and N-acetyl cysteine induced TFEB-dependent autophagy and NRF2 for protection against oxidative stress: implications for antioxidant supplements in neurodegeneration

Introduction

Aging, oxidative stress, and neurodegenerative diseases

Cells continually deal with abundant reactive oxygen species (ROS) generated by aerobic respiration or external factors. Hence, humans have evolved highly complex antioxidant systems (enzymatic and non-enzymatic) for counteracting ROS and oxidative stress (Circu & Aw, 2010; Ray et al., 2012). The enzymatic antioxidants, GSH-Px, catalase and superoxide dismutase (SOD), heme oxygenase 1 (HO-1), catalase and thioredoxin, are ubiquitously expressed by cells (Kurutas, 2016). The non-enzymatic antioxidants, which include Vitamin E and C and GSH are obtained from the diet or dietary supplements (Kurutas, 2016).

Aging and neurodegenerative diseases are associated with oxidative stress, including DNA damage and increased lipid and protein oxidation, due to the inadequacy of the endogenous antioxidant defense systems (Giordano et al., 2013; McBean et al., 2017). The levels of GSH, a principal endogenous thiol-based antioxidant, is particularly crucial in redox homeostasis in neurons and other specialized cells in the CNS (Aoyama & Nakaki, 2015). Its deficiency correlates strongly with neurodegenerative diseases (Mazzetti et al., 2015). In aging there is a lowering of GSH levels resulting from decreased synthesis (Y. Zhu et al., 2006), promoting oxidative stress.

Also, there is evidence to support aging-related deficiencies in vitamin C (ascorbate) and vitamin E (α-tocopherol) (Covarrubias-Pinto et al., 2015; Monacelli et al., 2017; Ulatowski & Manor, 2015). Overall, aging dysregulates the cellular antioxidant defense and, ultimately, the redox system, promoting oxidative stress and neurodegeneration.

Targeting autophagy and NRF2 for antioxidant defense

Autophagy and NRF2 (nuclear factor erythroid 2-related factor 2) are endogenous antioxidant pathways that regulate oxidative stress (de Vries et al., 2008; Vernon & Tang, 2013). NRF2 is a master antioxidant transcription factor, controlling the expression of a battery of cytoprotective genes and the enzymatic antioxidants, including GPx, HO-1, catalase and SOD, that counteract ROS production to maintain redox balance (Al-Sawaf et al., 2015; Filomeni et al., 2015; Moreno et al., 2018). The prerequisite for NRF2 activation is the disengagement of the NRF2 molecule from its repressor molecule Keap1, a ROS-dependent process that results in the modification of cysteine residues in Keap1 (Jiang et al., 2015; Silva-Islas & Maldonado, 2018).

Autophagy refers to the diverse lysosomal-centered pathways utilized in the degradation of intracellular substrates (Boya et al., 2013; Rubinsztein et al., 2011). Autophagy-targeted substrates include oxidized biomolecules (proteins, nucleic acids, carbohydrates, and lipids), damaged mitochondria, and other organelles (Filomeni et al., 2015). Substrates are degraded by three different routes; macroautophagy (hereafter referred to as autophagy), chaperone-mediated autophagy, and microautophagy (Boya et al., 2013; Galluzzi et al., 2017). Accumulation of toxic protein aggregates contributes to oxidative stress and the progression of neurodegenerative disorders, hence autophagy inhibits oxidative damage and neurodegeneration (Filomeni et al., 2015; Metaxakis et al., 2018). Due to the significant role of NRF2 and autophagy in modulating oxidative

stress and redox signaling, their compromise could be detrimental to cell survival and promote neurodegeneration (Giordano et al., 2013; Schmidt et al., 2015).

Role of antioxidants in neurodegenerative diseases

Among the limited therapeutic options for managing neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and AMD, antioxidant supplements, including vitamins C and E and the thiol-based antioxidants play an important role. (Gelain et al., 2012; Kumar & Singh, 2015; McBean et al., 2017). Since these vitamins and the thiol-based antioxidants are potent scavengers of ROS, there are concerns about their potential to dysregulate autophagy and NRF2 activation (Giordano et al., 2013; Schmidt et al., 2015; Underwood et al., 2010). This is because ROS are vital in signal transduction modulating autophagy and NRF2 (Filomeni et al., 2015; Schmidt et al., 2015; Vernon & Tang, 2013).

AMD is an important neurodegenerative disease of great public health interest due to its contribution to blindness and poor quality of life in the aging population (Wong et al., 2014). Antioxidant supplements are the mainstay treatment for the early stage of AMD (Mitchell et al., 2018), supported by the treatment outcomes from clinical trials reporting up to 25% reduction of AMD progression (Chew et al., 2012; Kassoff et al., 2001). In this study the effect of antioxidants on autophagy and NRF2 in human retinal pigment epithelial (RPE) cells was investigated, to help understand their role in the management of the AMD and other human neurodegenerative diseases.

It was found that the vitamin supplements and antioxidants, including vitamins C & E, N-acetyl cysteine (NAC), and GSH, induced autophagy in human RPE cells. Autophagy induction was associated with an upregulation of the transcription factor EB (TFEB), which regulates autophagy and lysosome biogenesis. In addition, the expression levels of p62 and the NRF2 antioxidant transcription factor were

upregulated, supporting the stabilization and activation of NRF2. The findings supported the role of autophagy and NRF2 in the protection of antioxidants against oxidative damage in human RPE cells. Thus, the neuroprotection effects of antioxidant supplements in AMD and neurodegenerative diseases may involve autophagy and NRF2 activation.

Methodology

Culture of ARPE-19 cells

Human RPE cells (ARPE-19, ATCC® CRL2302 ™) were cultured with commercially available Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen-Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin antibiotic mixture (Thermo Fisher Scientific, Rockford, IL, USA). Cells were incubated at 37ºC in a humidified atmosphere of 5% carbon dioxide. The growth medium was changed every three days until cells reached 90% confluency for use. Passages 16-25 were used for all experiments. According to the supplier (ATCC), the cells form stable monolayers, which exhibit morphological and functional polarity that could be carried for over 30 passages (Available:https://www.biocompare.com/20260-Miscellaneous-Human-Cell-Lines/875864-ARPE19/). Evidence also support that appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells (W. Samuel et al., 2017).

Cell viability assay and morphology

Briefly, ARPE-19 cells of seeding density $1x10^6$ cells/well was grown on a 6-well plate/35 mm MatTek glass-bottom dish (MatTek Corp., MA, USA) till ready for

experiment. Cells were then incubated with the designated doses of vitamin C (L-Ascorbic acid, A92902 Sigma-Aldrich), vitamin E (α-Tocopherol acetate, T3001, Sigma-Aldrich), glutathione (L-Glutathione reduced, G4251, Sigma-Aldrich) or N-acetyl cysteine (A9165 Sigma-Aldrich) for 24 h, followed by incubation with 50 µM hydroquinone (Sigma-Aldrich, H9003) or 800 µM H_2O_2 for 4 h (Hydrogen peroxide 30%, Merck Millipore # 1.07209). Treatments were performed in triplicate. For the viability, cells were trypsinized, centrifuged at 1500 rpm for 5 min and resuspended in the growth medium for counting. Briefly, the Trypan blue dye exclusion assay protocol used in determining cell viability involved trypsinization of cells, followed by centrifugation at 1500 rpm for 5 min and staining with 0.4% trypan blue solution (Sigma-Aldrich, T6146). Then, the ratio of viable cells to nonviable cells was computed using a hematocytometer chamber under a microspcope.

An assessment of the morphology of live cells grown in a 35 mm MatTek glass-bottom dish was performed using an inverted confocal microscopy (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam) and phase contrast with 10X magnification.

shRNA ATG5 or NFE2L2 knockdown

Stable knockdown of ATG5 in ARPE-19 cells was performed using lentivirus to deliver short hairpin RNA (shRNA). An aliquot of 3 x 10^6 HEK293T cells was seeded into a 10 cm culture dish. The cells were then transfected with lentiviral particles with either scrambled shRNA plasmid, ATG5 shRNA (TRC numbers: TRCN0000151474, Sigma-Aldrich) or shNFE2L2 shRNA (TRC numbers: TRCN TRCN0000007558, Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Scramble shRNA-coding lentiviral vector was purchased from Addgene (Addgene plasmid # 1864). After 8 h of transfection, the medium was removed and

replaced with fresh medium for another 48 h. Virions were collected and precipitated overnight using PEG, before filtering using a 0.45μm filter, and finally being transduced into ARPE-19 cells for 48 h. Cells were then subjected to puromycin (1.0 μg/mL) selection (Thermo Fisher Scientific, Inc.) for 10 days for the identification of resistant colonies.

Transfection of probe and fluorescence microscopy

ARPE-19 cells in a 6-well plate at about 90% confluency were transfected with 1 μg GFP-LC3-RFP-LC3ΔG (Addgene plasmid # 84572; RRID: Addgene_84572) using Lipofectamine 3000 (Thermo Fisher Scientific, L3000075) to monitor the autophagy flux. After transfection for 36 h, cells were incubated with the antioxidant supplements or vehicle for 24 h, to assess the effect of antioxidants on the autophagy flux. The medium was discarded and replaced with fresh medium for live-cell fluorescence imaging with an inverted microscope (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam, The Netherlands). Control and experimental groups were imaged with the same acquisition parameters. Quantification of fluorescence and intensity analysis of images was performed using ImageJ software.

Antioxidant treatment prior to protein/RNA extraction

Plated ARPE-19 cells were starved overnight in serum-free medium, followed by incubation of the cells with the designated dose of the antioxidant supplements (vitamin C, vitamin E, GSH, or N-acetyl cysteine) for 24 h. For the assessment of autophagy flux, co-incubation of cells with the designated dose of antioxidants and chloroquine (Chloroquine diphosphate salt, C6628, Sigma-Aldrich) was performed for 24 h. After treatment, cells were trypsinized (0.25% trypsin), pelleted by centrifugation at 1500

rpm for 5 min, washed twice with PBS, and stored at -80ºC for protein or RNA extraction.

Protein extraction and Immunoblotting for LC3-II, p62, NRF2, and LAMP-2A

Proteins were extracted using ice-cold 1X RIPA lysis buffer [0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA (Millipore)] containing 1:100 protease inhibitor cocktail (Thermo Scientific). Samples were sonicated for 1 h on ice at 4^0C, followed by centrifugation at 18000 x g for 30 min at 4^0C. The supernatant was obtained, and protein, concentration measured with BioRad Protein Assay (Bio-Rad).

A 30 µg aliquot of protein from each of the cell samples was loaded into wells of separating gel for SDS-PAGE electrophoresis (10% SDS-PAGE gels). All samples were denatured by the addition of β-Mercaptoethanol (M6250, Sigma-Aldrich) and incubation at 90ºC for 5 min before loading. Proteins were electrotransferred from gel to an Immobilon-FL PVDF membrane (Millipore) for 2 h at 250 mA using a cold pack and prechilled buffer to reduce the generation of heat. The membrane was then blocked using 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Bio-Rad Laboratories) for 1 h at room temperature. Primary antibody incubation with anti-TFEB (D2O7D, Cell Signaling Technology, 1:500), anti-LC3 (NB100-2220, Novus Biologicals, dilution 1:1000), anti-p62 (2C11, Novus Biologicals, dilution1:2000), anti-NRF2 (EP1808Y, Abcam, dilution 1:1000) or anti-LAMP-2 (sc-18822, Santa Cruz Biotechnology) were performed for 2 h at room temperature. Next, the membrane was washed three times for 10 min each time followed by incubation with horseradish peroxidase HRP-conjugated secondary antibodies from Thermo Fisher Scientific (anti-mouse IgG (H+ L), A16066, or anti-rabbit IgG (H+L), A16110; dilution 1:2000). The

membrane was again washed three times for 10 min. A mixed Enhanced chemiluminescence substrate solution was then added to the membrane, incubated for 5 min and immunoreactive bands imaged using ChemiDoc™ MP imaging system (Bio-Rad, 12003154). Quantification of bands was performed using ImageJ analysis software. Protein expression was normalized to GAPDH (AM4300, anti-GAPDH, dilution 1:2000, Thermo Fisher Scientific dilution).

Isolation of RNA, RT-PCR, and qPCR

Briefly, RNA extraction was performed using Trizol (Life Technologies) and following the manufacturer's protocol. RNA samples were free from contaminants; both the A260/280 and A260/230 ratios fell to within 1.9 to 2.1 (Thermo Scientific™ NanoDrop). Next, cDNA was reversed transcribed from 1 µg DNA-free total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and stored for conventional reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative PCR (qPCR).

For quantitative PCR, a PCR of reaction volume 10 μl per well was set up in triplicate using 2μl cDNA template, 5 μl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), 1 μl nuclease-free water, and 1 μl of gene-specific primers (Table 1). Quantitative RT–PCR was run on a LightCycler 480 System II (Roche Diagnostics). The reaction mixture was incubated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Fold changes were calculated using the change in the Cycle threshold (ΔΔCT) method.

For the conventional RT-PCR, PCR reaction volume of 20 µl was used containing the following: 1.5µl cDNA template, 10µl 2x Taq HS mix (R028A, Premix Taq™ DNA

Polymerase Hot-Start Version), 1 μl each of forward and reverse primers (10 µM) and 6.5 μl nuclease-free water. Amplification was performed for 25 or 28 cycles at a denaturing temperature of 94℃ for 30 s, annealing temperature of 60 °C for 30 s and an extension temperature of 72℃ for 30 s (MiniAmp Thermal Cycler, Applied Biosystems technology). The amplified products 10 μl PCR was mixed with 2µl 6× DNA loading buffer and DNA signal analyzed by 1.5% agarose gel electrophoresis containing GelRed stain (Biotium, Hayward, CA, USA) and visualized under UV light. β-actin or GAPDH reference gene was used for normalization of the expression of other genes.

Statistics

GraphPad Prism and Excel were used in analysing data and drawing graphs. All data are presented as mean ± SD. For one independent variable, the unpaired *t-test* was used for comparison of two groups and one-way ANOVA followed by Dunnett's multiple comparison *post hoc* tests performed when three or more groups were compared. Statistical significance was set at $p < 0.05$.

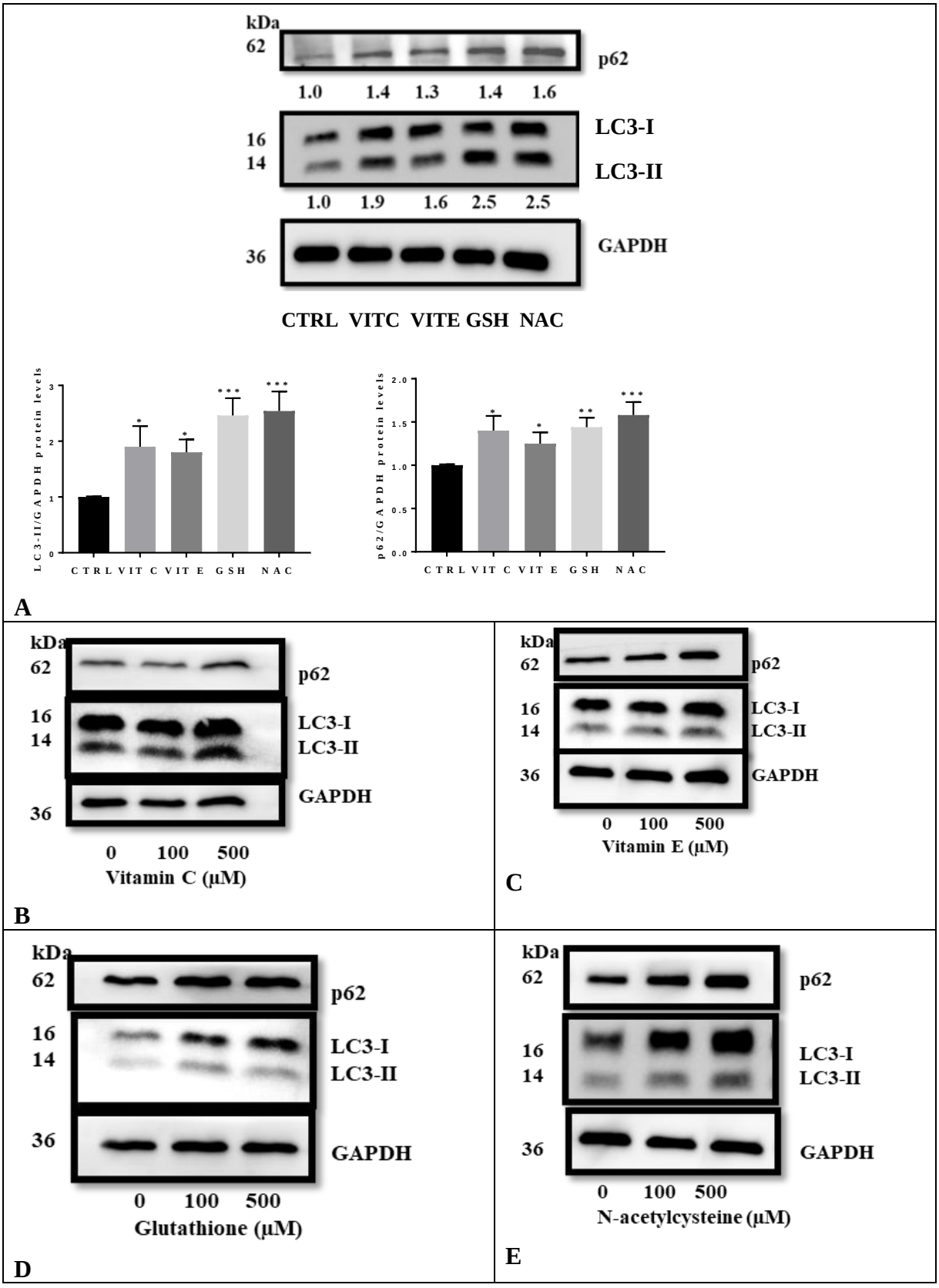
Results

Antioxidants increased LC3-II and p62 in human RPE cells

Autophagy is a catabolic process for the degradation of damaged intracellular proteins, lipids, DNA, and cellular organelles and the recycling of nutrients back for the synthesis of new molecules (Morel et al., 2017; Liang Wang et al., 2019). There is crosstalk between autophagy and phagocytosis (Ferrington et al., 2016). Dysregulation of autophagy in RPE cells, therefore, compromises phagocytosis, leading to the accumulation of photoreceptor outer segments and altering retinal proteostasis

(Ferrington et al., 2016). It was speculated that autophagy was involved in cytoprotection by antioxidant therapies in the management of AMD. Antioxidants recommended for AMD treatment include vitamins C & E, GSH, and NAC (Jennifer R. Evans & Lawrenson, 2017; Waugh et al., 2018). To investigate the effect of antioxidants on autophagy, changes in the microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) in cells incubated with the individual antioxidants for 24 h were monitored. LC3-II correlated with the number of autophagosomes due to the localization of LC3-II (or LC3-PE) to the autophagosome membrane after the cytosolic LC3 (LC3-I) conjugates with phosphatidyl-ethanolamine (PE) (Y. K. Lee & Lee, 2016). Each of the antioxidant supplements and the thiols increased LC3-II **(Fig. 4.1 A-I)**, suggesting enhanced autophagosome formation or blocked autophagosome turnover (Barth et al., 2010).

Changes in the autophagy receptor protein p62/SQSTM1, which is consumed when autophagy is upregulated (Bjørkøy et al., 2009), were also investigated. The antioxidant treatments also increased the p62/SQSTM1 levels **(Fig. 4.1 A-I)**. Although this outcome suggested that the autophagy flux was halted, previous studies had revealed that the p62 molecule could be increased in an autophagy-independent manner via transcriptional upregulation (Kuusisto et al., 2001; Sahani et al., 2014). Therefore, the findings of an increase in LC3-II and p62 protein levels by the antioxidants were not enough to determine their effects on the autophagy flux.


kDa
62
p62
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16
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LC3-I
LC3-II
1.0 1.9 1.6 2.5 2.5
36
GAPDH
CTRL VITC VITE GSH NAC
LC3-II/GAPDH protein levels
p62/GAPDH protein levels
CTRL VIT C VIT E GSH NAC
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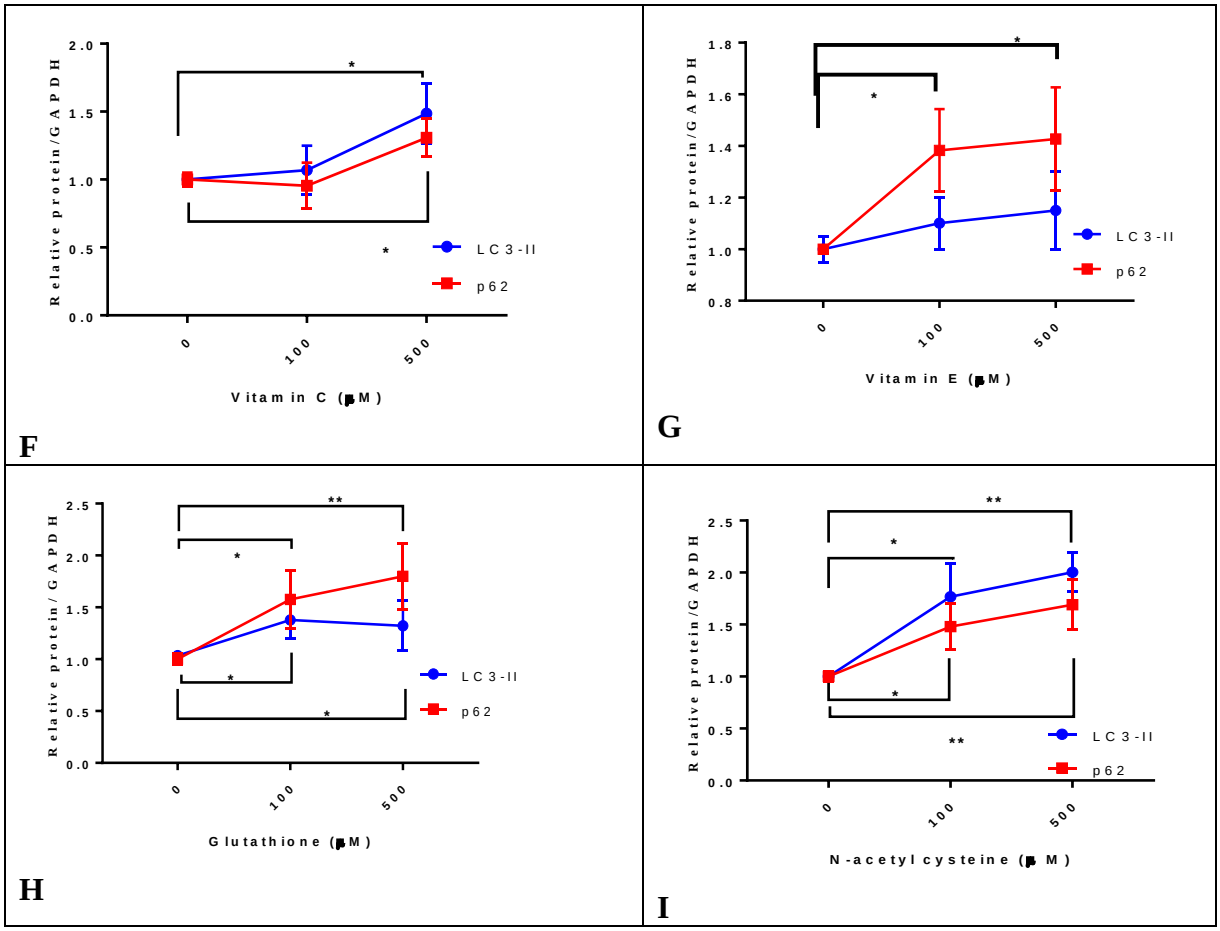


Figure 4. 1: Effect of antioxidants on autophagy marker LC3-II and p62.

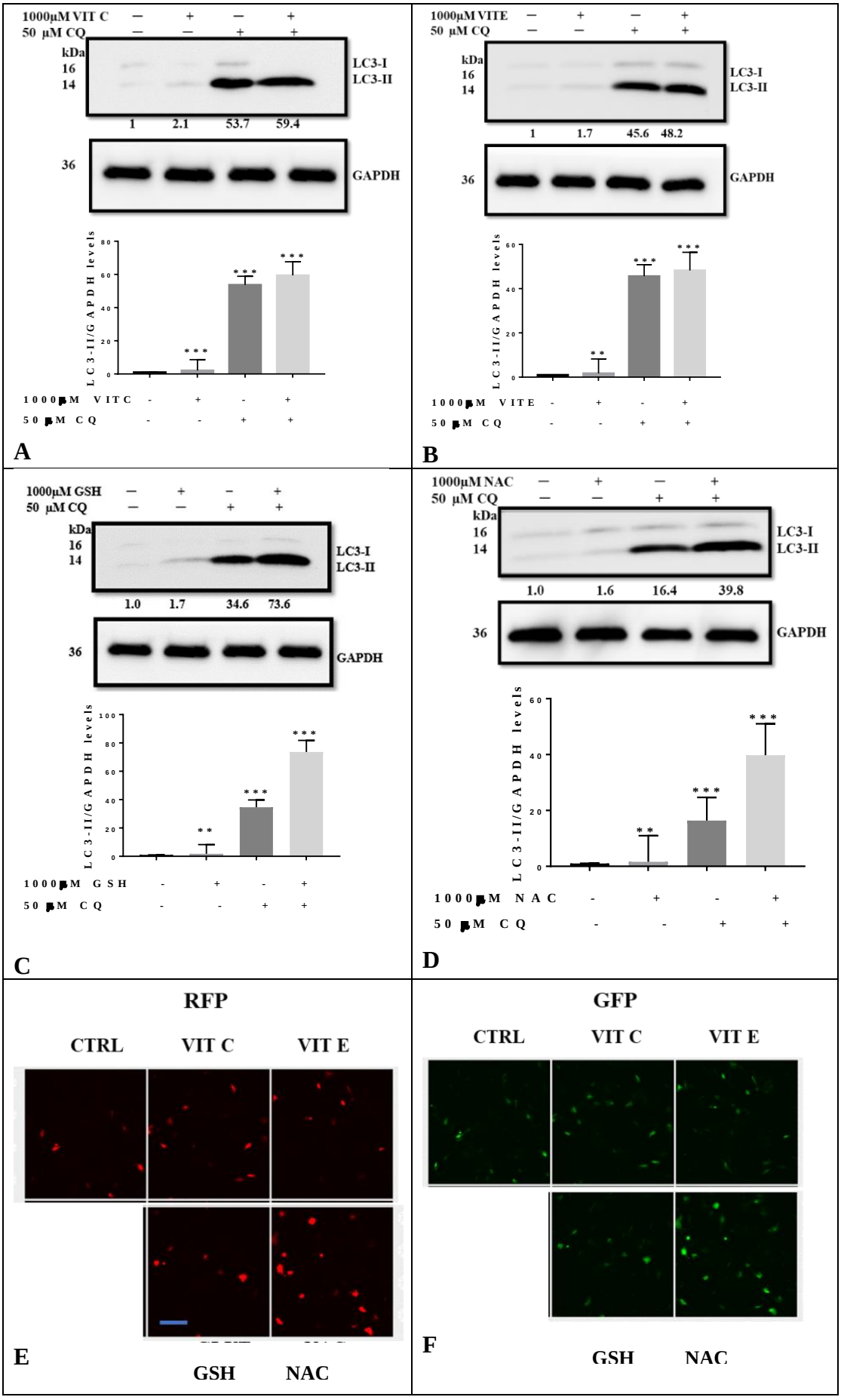
(A-E) Micrographs of immunoblots using whole-cell lysate from human RPE cells incubated with the designated dose of antioxidant for 24 h, run on SDS-PAGE, transferred onto PVDF membrane, and incubated with appropriate antibodies. (F-I) Densitometry quantification of protein levels normalized to GAPDH and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.01$ vs control.

Antioxidants upregulate autophagy flux in cells

An accurate assessment of the autophagy flux may be done by evaluating the differences in the amount of LC3-II between samples in the presence and without lysosomal protease inhibitors (Orhon & Reggiori, 2017). This is because LC3-II is itself degraded after autophagosome-lysosome fusion (Orhon & Reggiori, 2017). Hence, when a drug increases LC3-II in the presence of a lysosome inhibitor (e.g. chloroquine), it is an indication of autophagy induction by the drug (Mizushima & Yoshimori, 2007).

The results showed that the LC3-II level increased in cells incubated with vitamin C, vitamin E, GSH, or NAC in the presence of chloroquine (CQ) compared to CQ alone (**Fig. 4.2 A-D**), supporting autophagy induction by these antioxidants.

Furthermore, autophagy induction by antioxidants was monitored in cells transfected with the GFP-LC3-RFP-LC3ΔG, a novel fluorescent probe to evaluate autophagic flux (Kaizuka et al., 2016). This probe eliminates the artefact arising from the use of lysosome inhibitors (Kaizuka et al., 2016; Morishita et al., 2017). The probe is cleaved by endogenous ATG4 proteases into equimolar amounts of GFP-LC3 and RFP-LC3ΔG in the cell. GFP-LC3 is degraded by autophagy, while RFP-LC3ΔG remains in the cytosol, serving as an internal control. Thus, autophagic flux can be estimated by the GFP/RFP signal ratio (Kaizuka et al., 2016), a lowered GFP/RFP ratio indicating an increased autophagic flux. The results supported autophagy induction by antioxidants evident by the lower GFP/RFP fluorescence ratio and increased brown signal in the antioxidant-treated samples compared to the control (Fig. 4.2 E-H). This is because RFP is accumulated while GFP is depleted during autophagy upregulation (Morishita et al., 2017). The increase in the autophagy flux by vitamin E was, however, non-significant.


1000μM VIT C
50 μM CQ
kDa
16
14
LC3-I
LC3-II
1
2.1
53.7
59.4
36
GAPDH
LC3-II/GAPDH levels
1000μM VIT C
50 μM CQ
A
1000μM VITE
50 μM CQ
1
1.7
45.6
48.2
B
1000μM GSH
50 μM CQ
1.0
1.7
34.6
73.6
C
1000μM NAC
50 μM CQ
1.0
1.6
16.4
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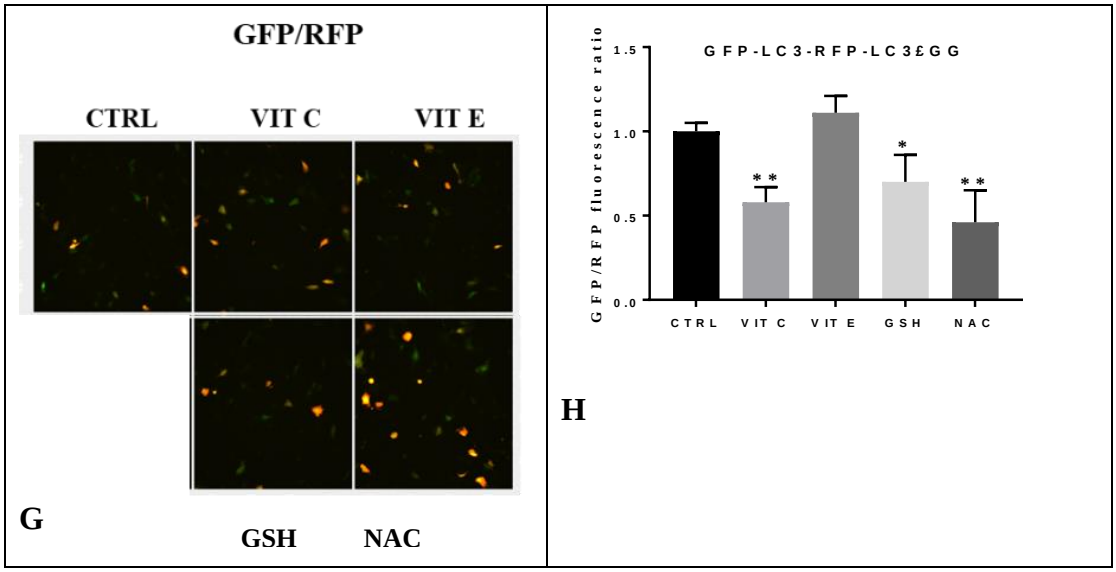


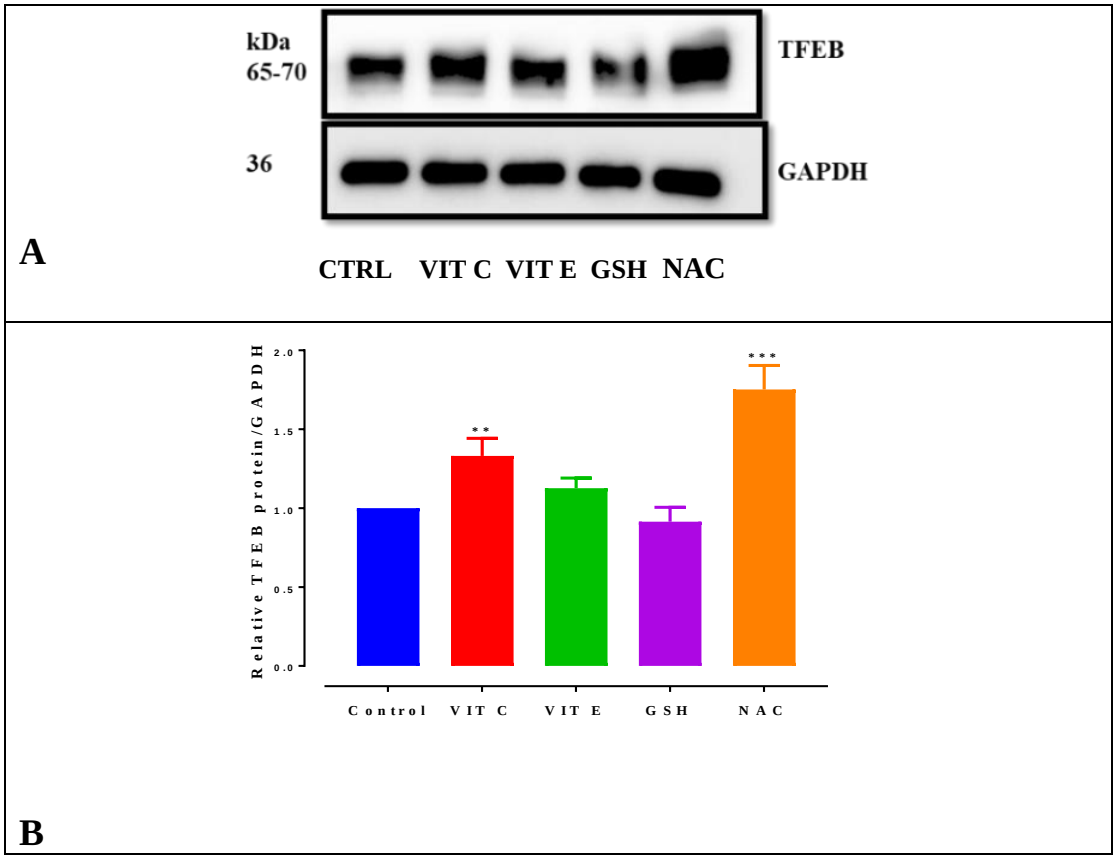
Figure 4. 2: Antioxidant supplements upregulate autophagy flux in RPE cells.

(A-D) Antioxidants supplements increased the autophagy flux. Immunoblotting analysis of whole-cell lysate from ARPE-19 cells co-treated with designated antioxidant and CQ or vehicle for 24 h. Densitometric analysis of protein expression was normalized with GAPDH. All expression levels are relative to the control. Data represent the mean ± SD of three independent experiments. (E–H) Reduced GFP-LC3 relative to RFP-LC3ΔG in cells incubated with antioxidant supplements. Wild-type ARPE-19 cells stably expressing GFP-LC3-RFP-LC3ΔG were incubated with antioxidants for 24 h and analyzed by fluorescence microscopy. Scale bar, 100 μm. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison *post hoc* tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ vs control.

Effect of antioxidants on TFEB, ATG5, and ATG7

Transcription factor EB (TFEB) is pivotal in the regulation of autophagy, as it promotes the expression of genes required for autophagosome formation, lysosome biogenesis, and lysosome function (Cortes & La Spada, 2019). Hence, to elucidate the mechanism involved in autophagy induction by the antioxidants, changes in TFEB mRNA and protein levels, as well as the mRNA level of autophagy-related genes ATG5 and ATG7, were investigated. The results supported TFEB-induction by vitamin C and NAC treatments, as cells incubated with these antioxidants showed elevated TFEB expression at both transcription and protein levels **(Fig. 4.3 A-C).** Vitamin E only increased the TFEB mRNA level (**Fig. 4.3 C**), but not its protein level compared to the control (**Fig. 4.3 A & B**). Transcriptional changes in TFEB levels may not always

translate at the protein level, since TFEB expression and activity is regulated at various levels by translational and post-translational modifications, such as acetylation and phosphorylation (H. Zhang et al., 2019; Jianbin Zhang et al., 2018). Hence, it is possible that vitamin E interferes with these mechanisms and needs further investigation. For the downstream TFEB target genes involved in autophagy, vitamin C was the only antioxidant upregulating mRNA levels of both ATG5 and ATG7 (Fig.4.3 C). Others upregulated either ATG5 or ATG7 (Fig.4.3 C). It is evident from the results that vitamin C and NAC are potent inducers of TFEB. For GSH, however, it is likely that a TFEB-independent autophagy pathway was involved, such as the transcription factor E3 (TFE3) activation (Martina et al., 2014) and other pathways connected to the mechanistic Target of Rapamycin complex 1 (MTORC1) (Ying Wang & Zhang, 2019).



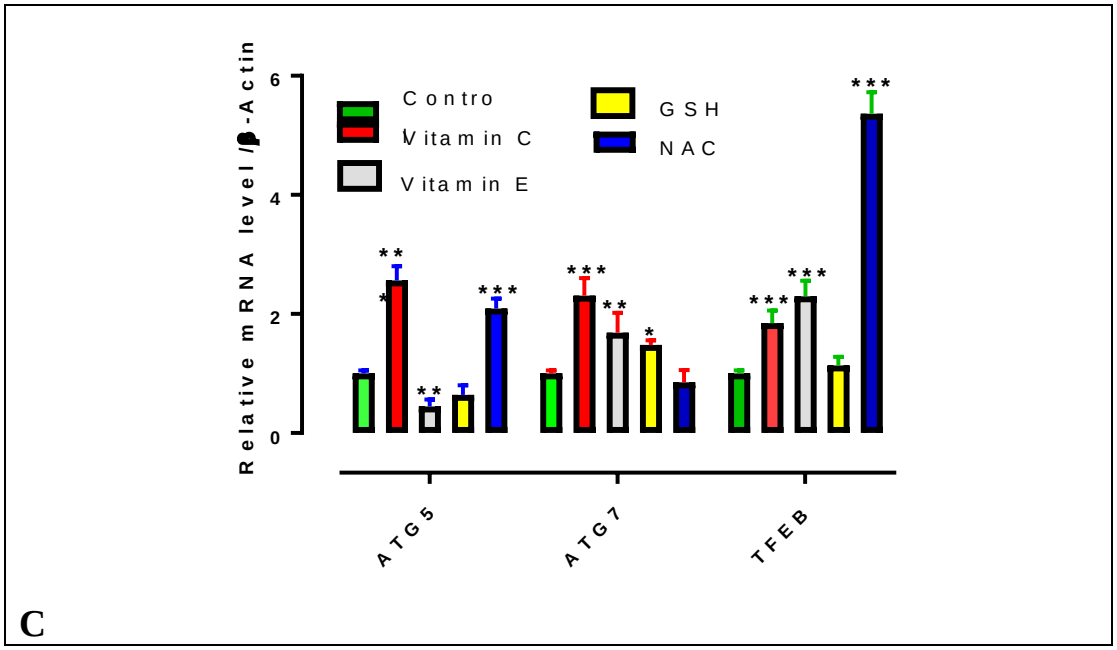


Figure 4. 3: Effect of antioxidant supplements on transcription factor EB (TFEB) and autophagy-related genes.

(A) Immunoblotting analysis of whole-cell lysate from plated ARPE-19 cells treated with 500 µM dose of antioxidant for 24 h. (B) Densitometric analysis of protein expression was normalized with GAPDH. (C) RNA extraction from the treated cells for real-time quantitative PCR. The mRNA expression level was normalized to β-actin and expressed as a ratio of the control. All expression levels are relative to the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by the one-way ANOVA followed by Dunnett's multiple comparison tests. $^{**}p < 0.01$, $^{***}p < 0.01$ vs control.

Effect of antioxidants on the chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is another important lysosomal pathway mediating the degradation of substrate, but is more selective compared to macroautophagy (H. Wu et al., 2015). Studies have shown that macroautophagy and CMA are closely linked to each other and regulated under stressful conditions, such as starvation and oxidative stress (H. Wu et al., 2015). Under persistently poor nutritional conditions due to prolonged starvation, CMA becomes upregulated to provide energy to cells while macroautophagy is downregulated (Cuervo, 2011). Lysosome-associated membrane protein (LAMP)-2A is the limiting factor for lysosomal degradation via CMA and, therefore, correlates directly with the rate of CMA activity (Susmita Kaushik

& Cuervo, 2018). The effect of antioxidants on CMA was investigated by assessing the changes in the expression of LAMP-2A in cells incubated with antioxidants. The results showed that except for GSH, all the antioxidants decreased LAMP-2A expression compared to the control (**Fig. 4.4 A-E**). These results suggest that most antioxidants promote macroautophagy at the expense of CMA inhibition, as the two pathways are interrelated (Susmita Kaushik & Cuervo, 2018; Massey et al., 2006).

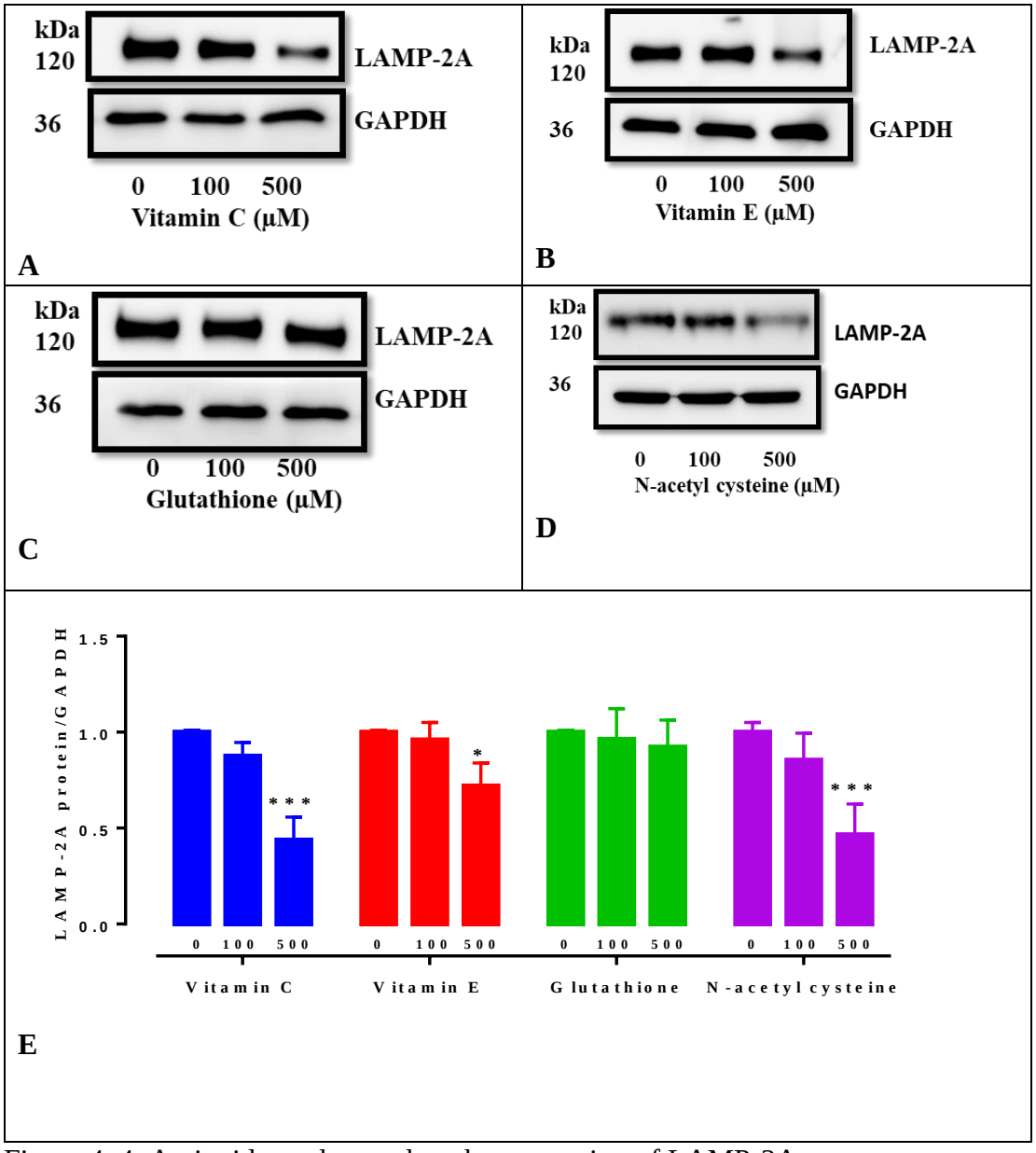
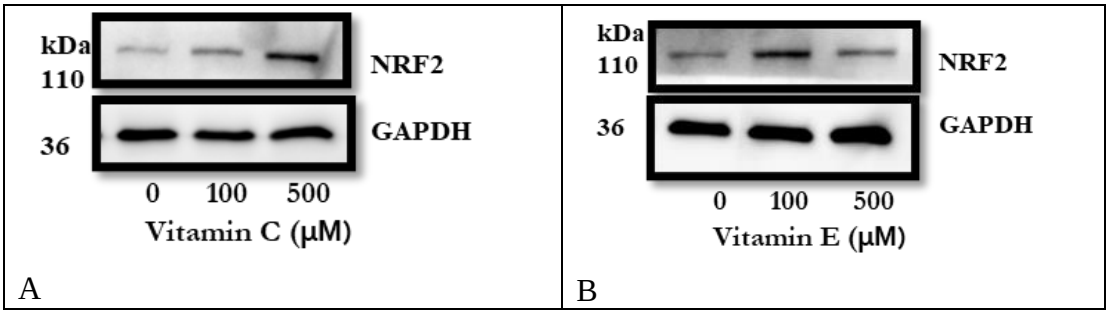


Figure 4. 4: Antioxidants dysregulate the expression of LAMP-2A.

(A-D) Human RPE cells were incubated with varying doses of antioxidant supplements or vehicle for 24 h, and whole-cell lysate prepared for SDS-PAGE and immunoblotting with LAMP-2A antibodies. (E) Densitometry quantification of LAMP-2 protein levels normalized to GAPDH and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by the one-way ANOVA followed by Dunnett's multiple comparison test. *$p < 0.05$, ***$p < 0.01$ vs control.

Antioxidants upregulate NRF2 protein expression in human RPE cells

NRF2 impairment is implicated in AMD, since RPE cells overlying retinal regions affected by AMD show a reduction in NRF2 protein levels (Lei Wang et al., 2014). ROS modulate signal transduction of the NRF2 antioxidant master transcription factor (Schmidt et al., 2015), leading to its stabilization and translocation into the nucleus, and activation of genes and antioxidant enzymes, including GPx, HO-1, SOD. (de Vries et al., 2008). Since antioxidants are generally known for scavenging of ROS, changes in NRF2 protein expression level were examined by western blot to determine whether the antioxidants impaired NRF2 activation in ARPE-19 cells. Contrary to the thought that antioxidants could impair NRF2 activation, the vitamins and thiol antioxidants upregulated NRF2 expression levels (**Fig. 4.5 A**-**E**). This finding is encouraging as NRF2 activation contributes in protection against oxidative stress (de Vries et al., 2008).



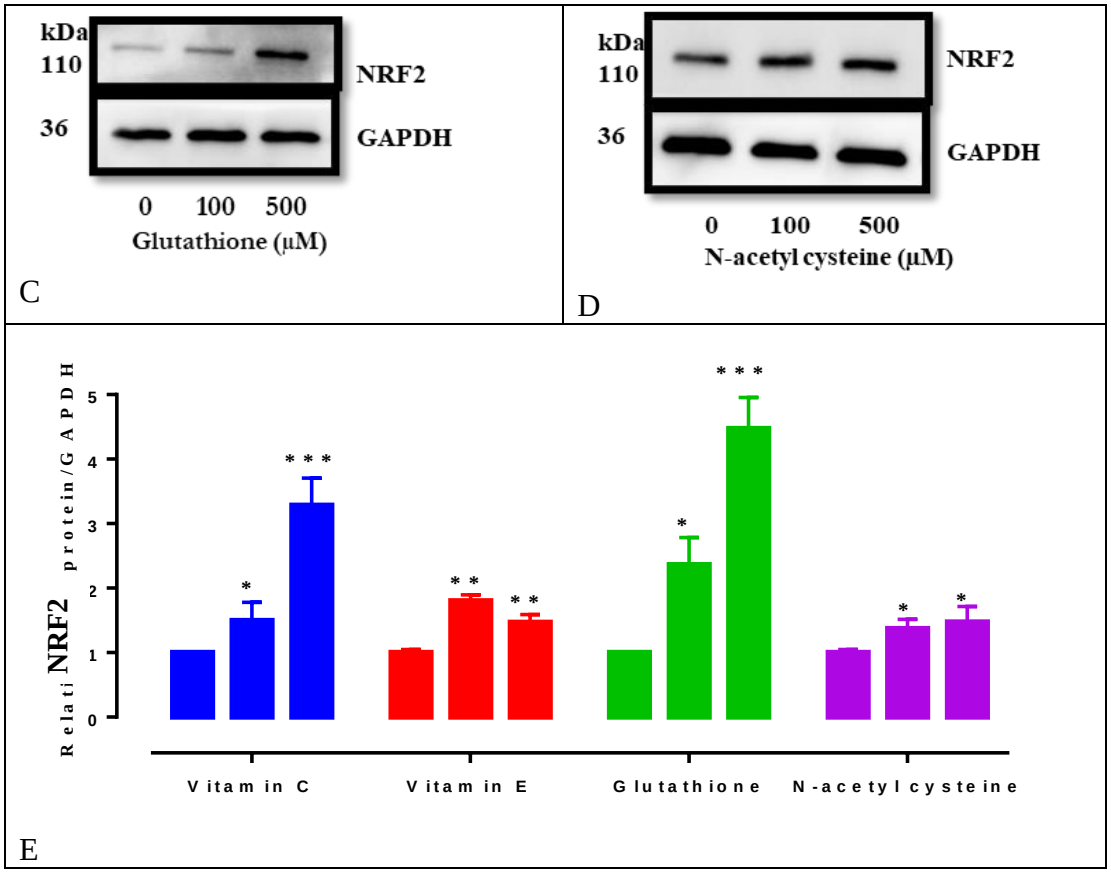


Figure 4. 5: Antioxidants upregulated NRF2 protein level in human RPE cells.

(A-D) Human RPE cells were incubated with varying doses of antioxidant supplements or vehicle for 24 h, and whole-cell lysate prepared for SDS-PAGE and immunoblotting with NRF2 antibody. (E) Densitometry quantification of NRF2 protein levels normalized to GAPDH and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by the one-way ANOVA followed by Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ vs control.

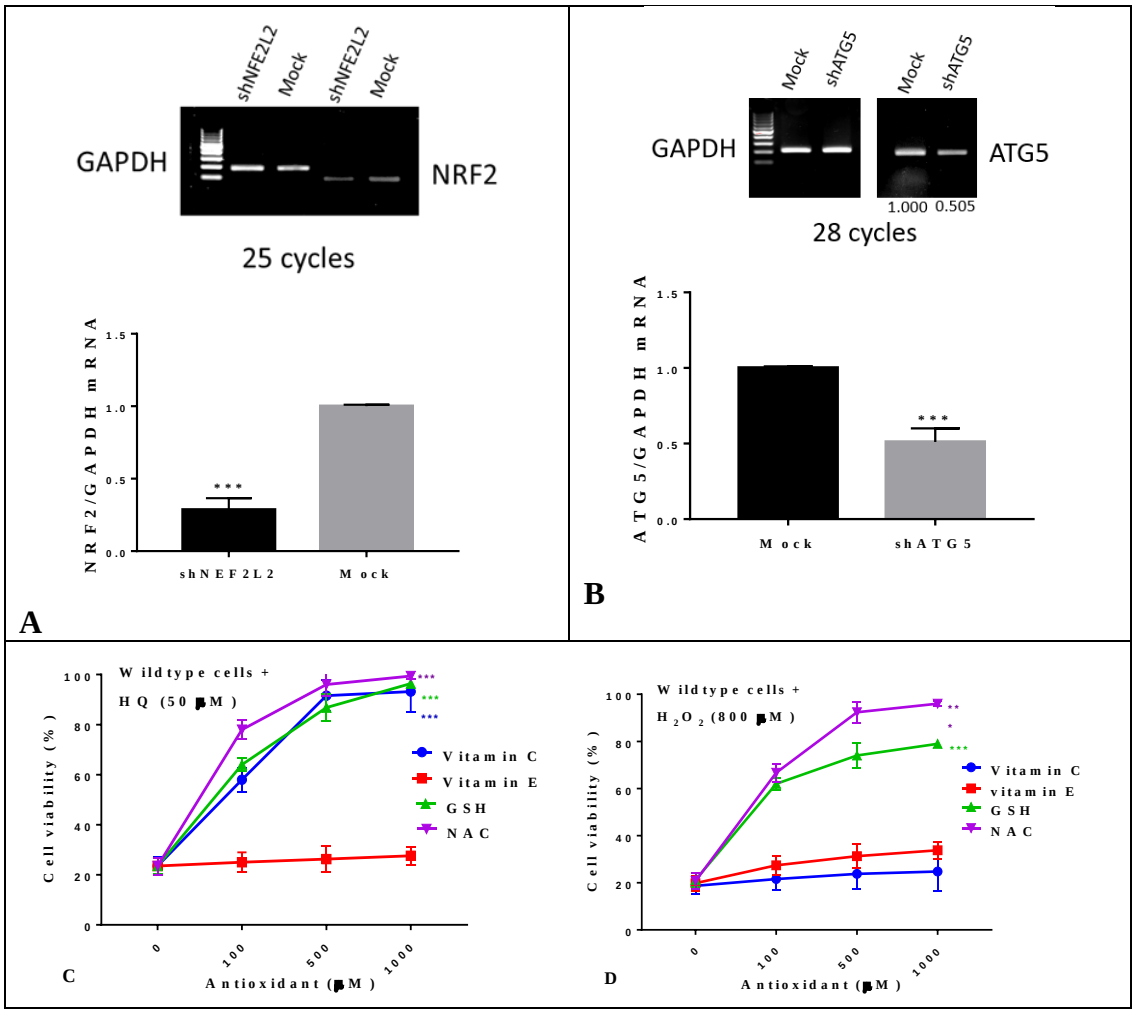
Evaluating the role of autophagy or NRF2 in the protection of RPE by antioxidants

It was hypothesized that autophagy or NRF2 was involved in protection against oxidative stress. Hence, the protective role of autophagy and NRF2 induction by antioxidant supplements on RPE cells under oxidative stress was evaluated by performing a dose-response assay for cell viability by the antioxidants in the presence of the oxidants hydroquinone (HQ) or H_2O_2. The dose-range of antioxidant vitamins and thiols (100, 500, or 1000 µM) used for treatment of cells in this study was chosen

based on their levels found in plasma and body tissues after oral supplementation, as reported in studies and clinical trials investigating the health effects of antioxidant vitamin supplementation (Flory et al., 2019; Hercberg et al., 2004; Jacob & Sotoudeh, 2002; Schmitt et al., 2015). In addition, reliable information from pharmacokinetic studies on the antioxidant vitamins and thiols that their bioavailability depends on the dose ingested was also considered, with the absorption rate ranging from 70%–90% for vitamin C (Jacob & Sotoudeh, 2002), 50–80% for vitamin E (Flory et al., 2019), and 55–70% for GSH (Buonocore et al., 2016).

Stable human RPE cells having defective NRF2 pathway or autophagy were generated by transfection of cells with the requisite shRNA plasmids to knockdown *ATG5* or *NFE2L2*. Results from RT-PCR confirmed significant inhibition of *NFE2L2* and the autophagy gene *ATG5* **(Fig. 4.6 A & B)**. The viability of cells (transfected with scrambled shRNA, shATG5, and shNFE2L2) before their incubation with any treatment (i.e., the control) ranged from 87 to 96% (graph not shown). The incubation of cells with the designated dose of HQ or H_2O_2 without antioxidant pretreatment caused marked reduction in the viability of approximately 20% (**Fig.4.6 C-H**). A 4-h incubation period was chosen to allow comparison of results with earlier studies investigating RPE protection from oxidative damage by other antioxidants (Neal et al., 2020). In the RPE cells transfected with scrambled shRNA (referred as the wildtype), all the antioxidant pretreatments showed significant cytoprotection against HQ and/or H_2O_2, except for vitamin E (Fig. 4.6 C & F; **one-way ANOVA; $p < 0.001$**). In a similar fashion, although unexpected, the impaired NRF2 cells pretreated with antioxidants were also protected from HQ and/or H_2O_2 toxicity (**Fig. 4.6 D & G, one-way ANOVA, $p < 0.001$**), even though the protective effect was lower compared to that achieved in the wildtype. This suggests that NRF2 activation was involved, but not essential in the

cytoprotection by the antioxidants against oxidative damage from HQ and H_2O_2. On the contrary, no antioxidant pretreatment could rescue the shATG5 knockdown cells from H_2O_2 -induced toxicity (**Fig. 4.6 E; one-way ANOVA; *p* > 0.05**), and the antioxidants also showed lower protection against HQ-toxicity (**Fig. 4.6 H, one-way ANOVA, *p* < 0.001**). Specifically, the results clearly showed that autophagy induction by GSH and NAC was essentially involved in their cytoprotection against H_2O_2. Because the antioxidants activated both autophagy and NRF2, RPE protection was still observed even when one of the antioxidative pathways was compromised. Collectively, these findings emphasize the crucial role of autophagy and NRF2 activation by antioxidants in their protection against various oxidants in human RPE cells.



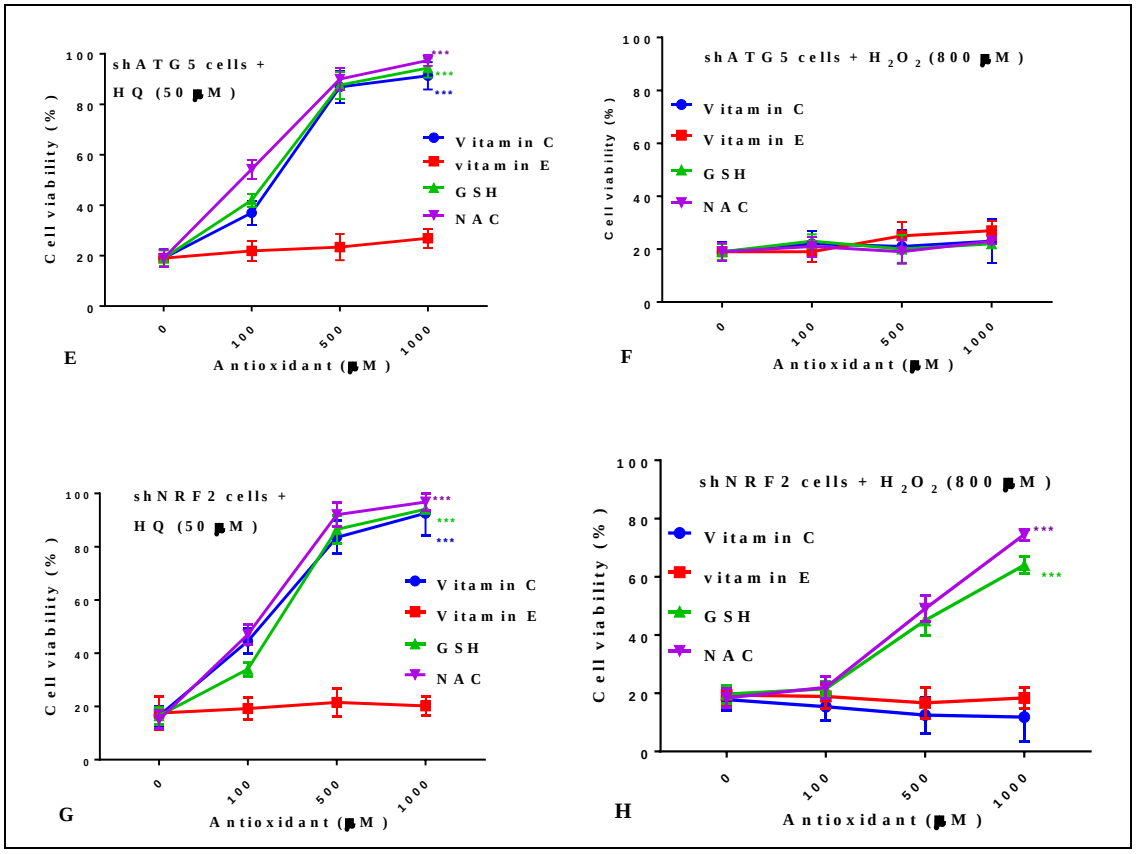


Figure 4. 6: Inhibition of autophagy or NRF2 lowered or abolished the cytoprotection by antioxidants.

(A-H) ARPE-19 cells transfected with scrambled, shRNA ATG5, or shNFE2L2 were treated with vitamins C and E, glutathione (GSH), or N-acetyl cysteine (NAC) for 24 h prior to contact with H_2O_2 or hydroquinone (HQ) for 4 h. (A & B) Reverse transcription PCR was used to validate the knockdown of *ATG5* or *NFE2L2* in cells. (C−H) Trypan blue assay was used to quantify cell viability or cytoprotection of antioxidants against HQ or H_2O_2. Data represent the mean ± SD of 3 independent experiments. The unpaired *t* test or one-way ANOVA followed by Dunnett's *post hoc* test was performed to determine statistical significance. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$.

Discussion

Autophagy and NRF2 are essential cellular antioxidant pathways modulating oxidative stress and redox homeostasis (Giordano et al., 2013; Schmidt et al., 2015; Underwood et al., 2010). Therefore, evaluating the risk associated with the dysregulation of autophagy or NRF2 by an existing therapeutic intervention for managing neurodegenerative diseases is important. As antioxidant supplements are one of the limited treatment choices for managing AMD and other neurodegenerative diseases

(McBean et al., 2017; Schmidt et al., 2015), their effect on human RPE cells, the primary site of oxidative injury in AMD (Datta et al., 2017; Kinnunen et al., 2012) was investigated. The selection of vitamins C and E, GSH, and NAC was based on the evidence of efficacy and their widespread use in the management of AMD and other neurodegenerative diseases (Gelain et al., 2012; Gil-Martínez et al., 2020; Kumar & Singh, 2015; McBean et al., 2017). The role of autophagy and NRF2 induction by antioxidants in the protection against oxidative stress was also investigated. The novel findings in this study are that not only do antioxidant supplements, including vitamin C, vitamin E, glutathione, and N-acetyl cysteine, upregulate the autophagy flux and NRF2, but these two mechanisms contribute to their cytoprotection against oxidative stress. Based on the therapeutic potential of autophagy and NRF2 activation in neurodegenerative diseases (Giordano et al., 2013; McBean et al., 2017), determining that the antioxidant supplements provide these additional benefits is important.

The concerns about the risk of dysregulating redox homeostasis by antioxidants with the capacity of scavenging free radicals is premised on the vital role of ROS in modulating the signal transduction of autophagy and NRF2 (Filomeni et al., 2015; Poljsak et al., 2013; Schmidt et al., 2015; Vernon & Tang, 2013). However, studies on the effect of antioxidants on autophagy or NRF2 report gives inconsistent results (Karim & Kadowaki, 2017; Martin et al., 2002; Underwood et al., 2010; F. Zhao et al., 2017). Rubinsztein and his team reported the inhibition of basal autophagy and the promotion of neurodegeneration in models of polyglutamine disease by antioxidant supplements, including thiols (NAC, GSH, and cystamine) and vitamin E (Underwood et al., 2010). In their study, they showed that the thiol antioxidants could inhibit autophagy in a ROS-independent manner, based on the assessment of LC3-II changes

and the degradation of alpha-synuclein in transfected HeLa cells (Underwood et al., 2010).The current study's results are in stark contrast, as they took advantage of dual fluorescence probes and convincingly demonstrated that antioxidant supplements, particularly NAC and vitamin C, were potent autophagy inducers in RPE cells. More recent reports corroborated autophagy induction by vitamin C supplements in human liver cells, with a concentration lower than the physiological level (Karim & Kadowaki, 2017; F. Zhao et al., 2017). Martin et al. found that vitamin C upregulated both intracellular and extracellular degradation of proteins in glial cells, via lowering and stabilization of the acidic lysosomal pH and induction of lysosomal hydrolases (Martin et al., 2002).

Until recently, accurate monitoring of autophagy was challenging, requiring a battery of tests due to the dynamic and multistage nature of autophagy (Klionsky et al., 2016; Mizushima et al., 2010). The problems of these earlier methods have been extensively reviewed (Klionsky et al., 2016). Although the methods for evaluation of autophagy flux with lysosomal inhibitors are accepted, lysosomal inhibition is limited by unwanted secondary effects on mTORC1 or LC3-II formation (Florey et al., 2015). Therefore, in addition to the assessment of autophagy flux with chloroquine, the present study used a new fluorescent probe GFP-LC3-RFP-LC3ΔG for investigating autophagy induction by the antioxidant supplements (Kaizuka et al., 2016). This probe was designed to overcome the shortcomings associated with using lysosomal inhibitors and does not require any control sample for comparison (Kaizuka et al., 2016; Morishita et al., 2017). This is because the levels of GFP and RFP intensities are compared within each sample to determine whether any difference can be found. Higher levels of RFP compared to GFP is indicative of increased autophagy flux since GFP is degraded during autophagy (Kaizuka et al., 2016; Morishita et al., 2017). The autophagy flux

results of the probe were consistent with the autophagy flux measurement in the presence of chloroquine, providing additional support for autophagy induction by vitamins C, GLUT and NAC.

The findings data showed that NAC and vitamin C upregulated TFEB at the transcription and protein levels, supporting autophagy induction by these antioxidants. TFEB inducers are recognized as potent autophagy induction and neuroprotection agents (Cortes & La Spada, 2019; Napolitano & Ballabio, 2016). TFEB overexpression promotes autophagosome formation, biogenesis of new lysosomes, and lysosomal enzymes for degradation (Sardiello et al., 2009; Settembre & Ballabio, 2011). This coordinated upregulation of various steps in autophagy by TFEB is mediated by the activation of the Coordinated Lysosomal Expression and Regulation (CLEAR) gene network (Sardiello et al., 2009; Settembre & Ballabio, 2011). In addition to autophagy, TFEB activation of the CLEAR gene network promotes cellular catabolism of lipids, glucose homeostasis, and mitochondrial biogenesis (Sardiello et al., 2009). These functions of TFEB induction justifies the protective role of TFEB inducers in models of neurodegenerative diseases, including Parkinson's disease (PD), Huntington's disease (HD), and AMD, with defective autophagy (Cortes & La Spada, 2019; Martini-Stoica et al., 2016). The results of the present study indicated that GSH downregulated TFEB expression and upregulated autophagy. It is, therefore, possible that a TFEB-independent autophagy pathway was activated, such as the transcription factor E3 (TFE3) activation (Martina et al., 2014), inhibition of MTORC1, or other upstream autophagy pathways (Ying Wang & Zhang, 2019). As a result, caution may be needed in prolonged GSH supplementation. Also, since GSH is a tripeptide (comprising of three amino acids cysteine, glutamic acid, and glycine) it is possible the cells rather

absorbed these constituent amino acids, altering the nutrient sensing and mTOR pathways and resulting in the inhibition of TFEB (Bar-Peled & Sabatini, 2014). Further investigation is needed to elucidate the impact of GSH supplementation on the TFEB expression levels.

Vitamin E did not change the TFEB protein levels at the tested concentrations, suggesting that other pathways might be involved in the autophagy upregulation. A recent study demonstrated that higher doses of vitamin E alleviated nephropathy in streptozotocin-induced diabetic rats by enhancing autophagy (Y. Zhao et al., 2019). Possibly the insignificant increase observed in TFEB protein level and autophagy during vitamin E supplementation accounted for the lack of cytoprotection. In addition to increasing the dose, the availability of fat is an important determinant for vitamin E absorption into cells, since it is a liposoluble vitamin (Flory et al., 2019).

The antioxidants upregulated NRF2 transcription factor in cultured human RPE cells, supporting NRF2 stabilization and activation. Under normal conditions, the NRF2-Keap1 complex in the cytosol is ubiquitinated and degraded by the proteasome (Kobayashi et al., 2004). NRF2 activation via the canonical pathway necessitates an increase in ROS levels, to cause the modification of cysteine residues of the Keap1 repressor and lead to NRF2 stabilization (Kobayashi et al., 2004). The upregulation of the NRF2 by antioxidants may involve a different mechanism independent of ROS, referred to as the non-canonical pathway (Katsuragi et al., 2016; Silva-Islas & Maldonado, 2018). This is a p62-mediated NRF2 activation pathway and has been well documented in autophagy defective cells, where there is an accumulation of the autophagy substrate p62 (Katsuragi et al., 2016; Komatsu et al., 2010). p62 sequesters

Keap1 to allow stabilization of NRF2 and translocation into the nucleus (Katsuragi et al., 2016; Komatsu et al., 2010). As the p62 level was increased in the antioxidant treated cells with competent autophagy, the non-canonical activation of NRF2 may be enhanced.

Conclusion

The present study revealed a novel protective mechanism through which antioxidant vitamins supplementation and N-acetyl cysteine induced cytoprotection that includes the upregulation of TFEB, master regulator of lysosomal biogenesis, and downstream autophagy genes. The vitamins and thiol-based antioxidants upregulated autophagy and NRF2 antioxidant pathways for protection against oxidative damage in human RPE cells. These findings show that antioxidant supplements have a greater impact on modulating redox homeostasis beyond their basic free-radical scavenging role. Thus, TFEB overexpression, autophagy, and NRF2 activation may play a crucial role in the protective effects of antioxidant vitamin supplements and thiols in neurodegenerative diseases.

CHAPTER V

Autophagy upregulation by the TFEB-inducer trehalose protects against oxidative damage and cell death associated with NRF2 inhibition in human RPE cells

Introduction

The etiology of age-related macular degeneration (AMD) is multifactorial and includes both genetic and environmental risk factors (Cheung et al., 2017; N. G. Lambert et al., 2016). Oxidative damage to the retinal pigment epithelium (RPE), however, appears to play a crucial role based on *in vivo* studies in AMD subjects and animal models of retinal degeneration, as well as *in vitro* cell culture models of AMD (Abokyi et al., 2020). The increased risk of developing AMD among cigarette smokers and the intimate relationship between the number of pack-years of smoking and disease progression are compelling evidence implicating oxidative stress in AMD (Joachim et al., 2018; Myers et al., 2014). Experimental studies further our understanding of the association between smoking and AMD by demonstrating that the RPE is susceptible to oxidative damage upon exposure to cigarette smoke or its pro-oxidant hydroquinone (HQ) (Espinosa-Heidmann et al., 2006). In mice, it was found that prolonged exposure to cigarette smoke damaged the RPE and led to AMD-like retinal changes (Espinosa-Heidmann et al. 2006). These findings elucidate the primary role of oxidative RPE damage in the development of AMD.

Nuclear factor erythroid 2-related factor 2 (NRF2) activation is a master antioxidant transcription factor that regulates oxidative stress (Robledinos-Antón et al., 2019; Tebay et al., 2015). Under normal basal conditions, the NRF2 antioxidant transcription

factor is bound to the Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol, and its level is tightly regulated via the ubiquitin-proteasome system (UPS) (Robledinos-Antón et al., 2019). The activation of NRF2 occurs when it disassociates from the KEAP1 repressor. Consequently, NRF2 stabilizes and translocates into the nucleus leading to the activation of the antioxidant response elements (ARE) for the induction of detoxifying (phase II enzymes) and antioxidant enzymes (Tonelli et al., 2018). Hence, NRF2 activation under oxidative stress protects against oxidative damage and promotes cell survival. However, post-mortems conducted on specimens from eyes with AMD have shown that the NRF2 antioxidant transcription factor was downregulated in RPE cells overlying drusen (Lei Wang et al., 2014). Studies have reported that the major risk factors of AMD, including aging and cigarette smoking, promote oxidative damage in RPE cells by inhibition of NRF2 antioxidant defense (Cano et al., 2010; Sachdeva et al., 2014). In aged rats there is the inhibition of NRF2 mRNA, reduced antioxidant enzymes, and increased oxidative stress in the RPE, promoting $NaIO_3$-induced retinal degeneration (Sachdeva, Cano, and Handa 2014). We also observed that HQ depleted NRF2 and increased oxidative damage in RPE cells *in vitro*, consistent with the effect of cigarette smoking on primary bronchial epithelial cells of patients with chronic obstructive pulmonary disease (COPD) (Yamada et al., 2016). Hence, we proposed that since oxidative damage of the RPE and AMD was associated with NRF2 inhibition, targeting an alternate robust antioxidant pathway could be an effective approach to protect against RPE damage and AMD.

Autophagy is a catabolic mechanism involving lysosomal degradation of cytosolic material, including macromolecules and organelles. In mammals, three autophagic pathways target substrates for lysosomal degradation (Morel et al., 2017).

Macroautophagy involves the formation of autophagosomes, double-membrane vesicles, to transport substrates to lysosomes for degradation. In chaperone-mediated autophagy (CMA), lysosomal degradation is regulated by the interaction between lysosome-associated membrane protein 2 (LAMP2), a lysosomal receptor, and the chaperone Hsc70, facilitating the selective access of substrates with the target motif (KFERQ) into the lysosome. Lysosomal degradation by microautophagy is the simplest, involving the direct invagination and sequestration of substrates into the lysosome (Morel et al., 2017). Studies of neurodegenerative diseases linked to oxidative stress, such as Alzheimer's, Parkinson's, Huntington's disease, amyloid lateral sclerosis, and AMD, revealed that autophagy induction conferred cytoprotection (Kaarniranta et al., 2013; Mitter et al., 2014). Based on the emerging role of autophagy in neurodegeneration, the inducers of transcription factor EB (TFEB), a major regulator of autophagy and lysosomal biogenesis, have received considerable attention (Cortes and La Spada 2019; Martini-Stoica et al. 2016). TFEB coordinates multiple steps in the autophagy-lysosomal pathway via the activation of the coordinated lysosomal expression and regulation (CLEAR) network of genes (Settembre and Ballabio 2011).

Recently, a small molecule called trehalose, a natural existing disaccharide of known neuroprotection against numerous neurodegenerative disease models (Emanuele, 2014; Hosseinpour-Moghaddam et al., 2018), was found to be a potent TFEB inducer in neurons (Lotfi et al., 2018; Rusmini et al., 2019a). Trehalose is safe and approved as a food ingredient for human consumption by the European regulatory system and the U.S. Food and Drug Administration in 2000 (Richards et al., 2002). This disaccharide is in use for topical and systemic treatment of ocular and systemic disorders, including oculopharyngeal muscular dystrophy, spinocerebellar atrophy type 3, atherosclerosis,

fatty liver disease, dry eyes, and in the post-surgical management of laser-assisted in situ keratomileusis (LASIK) in multiple clinical trials (Argov et al., 2015; Chen et al., 2009; Mateo et al., 2017; Noorasyikin et al., 2020),(https://clinicaltrials.gov/ct2/show/NCT03700424;https://clinicaltrials.gov/ct2/show/NCT03738358). While autophagy upregulation by trehalose is well reported in many studies (Khalifeh et al., 2019; Rusmini et al., 2019), a few reports have disputed this claim (Tien et al., 2016; Yoon et al., 2017). In this study, autophagy induction by trehalose on human RPE cells was investigated. Also, its cytoprotective role against hydroquinone, an oxidant that impairs the NRF2 transcription factor, was explored. The results support trehalose as a unique autophagy inducer in the human RPE cells. The disaccharide upregulated autophagy flux and the mRNA or protein levels of key autophagy regulators, including TFEB, ATG5 and ATG7, LC3-II, LAMP2, and the lysosomal hydrolase cathepsin D. Collectively, the evidence supports an upregulation of crucial stages in the autophagy-lysosomal pathway, such as 1) the initiation of autophagosome 2) autophagosome formation and 3) autophagosome maturation, autolysosome formation, and substrate degradation. Also, the potential therapeutic benefits of trehalose in AMD was ascertained by demonstrating its autophagy-dependent cytoprotection against the cigarette smoke oxidant HQ in human RPE cells.

Methodology

Culture of ARPE-19 cells

The human RPE cells (ARPE-19, ATCC® CRL2302 ™) were cultured with the commercially available Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen-Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin antibiotic mixture (Thermal

Fisher Scientific, Rockford, IL, USA). Cells were incubated at 37ºC in a humidified atmosphere of 5% carbon dioxide. The medium was changed every 3 days until cells reached 90% confluency for use in experiments. Passages 16-25 were used for experiments. According to the supplier (ATCC), the cells form stable monolayers, which exhibit morphological and functional polarity that could be carried for over 30 passages (Available:https://www.biocompare.com/20260-Miscellaneous-Human-Cell-Lines/875864-ARPE19/). Evidence also support that appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells (W. Samuel et al., 2017).

Cell viability assay and morphology

In brief, ARPE-19 cells of seeding density $1x10^6$ cells/well were grown on a 6-well plate/35 mm MatTek glass-bottom dish (MatTek Corp., MA, USA) until ready for experiment. Cells were then incubated with the appropriate doses of trehalose (Sigma-Aldrich, T9449) or incubated with 50 µM HQ (hydroquinone, Sigma-Aldrich, H9003) or vehicle. Each treatment was performed in triplicate. Cells were either detached with trypsin and stained for Trypan blue dye exclusion assay or imaged using an inverted confocal microscopy (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam) and phase contrast with 20X magnification as described earlier.

CM-H2DCFDA assay for intracellular ROS

Cells ($5x10^4$ cells/well) were transferred into 96-well plates for incubating overnight, followed by incubation with 0-100 mM trehalose in serum-free medium for 24 h. The medium was then removed, the cells washed with PBS, and incubated with 5 µM of *5*-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate CM-H2DCFDA (C6827, Invitrogen) in the dark at 37°C for 1 h. On uptake into the cells, the probe is

oxidized by intracellular ROS converting it from its non-fluorescent state to a fluorescent form. Fluorescence intensity was measured at 483 nm against 530 nm as reference using a Clariostar microplate reader (BMG Labtech, Offenburg, Germany).

Determination of Protein carbonyl level by ELISA

Protein carbonyl concentration was measured in protein samples using the Oxiselect™ protein carbonyl ELISA kit (Cell Biolabs, STA-310) according to the manufacturer's instructions. Briefly, the protein samples prepared from cells preincubated with trehalose or vehicle before exposure to HQ were incubated with 1% streptomycin sulfate (Sigma-Aldrich, S9137) and diluted to the specified 10µg/ml protein concentration. Protein samples were then adsorbed onto a 96-well plate for 2 h at 37oC and derivatized to dinitrophenylhydrazine (DNPH). After treating with anti-DNP antibody and followed by Horseradish Peroxidase (HRP) conjugated secondary antibody. The absorbance was measured at 450 nm wavelength using a microplate reader (Ao, Azure Biosystems Inc., Dublin, USA).

Proteasome Activity Assay

Proteasome activity was measured using a fluorogenic 7-amino-4-methyl coumarin (AMC)-tagged substrate kit to detect chymotrypsin-like activity, following the manufacturer's protocol (Biovision proteasome activity assay kit, San Francisco, CA). Briefly, ARPE-19 cells incubated with trehalose or vehicle for 24 h, were lysed with 25mM Tris-HCl buffer for 60 min at 4 °C, and the supernatant collected by centrifugation at 13,000 rpm for 10 min at 4 °C. The cell lysates of samples were loaded onto a 96-well plate in duplicate for incubation either with the fluorescent substrate at 37 °C for 30 min in the presence of MG132 (proteasome inhibitor) or without (as

control). The proteasome has chymotrypsin-like which releases free, highly fluorescent AMC from the AMC-tagged peptide substrate. Fluorescence intensity was then measured at 350 nm against 440 nm as reference using a Clariostar microplate reader (BMG Labtech, Offenburg, Germany). Protein concentration was used for the normalization of the data.

shRNA knockdown of ATG5

Stable knockdown of ATG5 in ARPE-19 cells was performed using lentivirus to deliver short hairpin RNA (shRNA). An aliquot of 3 x 106 cells of HEK293T cells was seeded into 10 cm culture dishes. The cells were then transfected with lentiviral particles with either scrambled shRNA plasmid or ATG5 shRNA, TRC numbers: TRCN0000151474 (Sigma Alrich) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. A scramble shRNA-coding lentiviral vector was purchased (Addgene plasmid # 1864). After 8 hours, the medium was changed, and incubation continued for another 48 hours. Virions were collected and precipitated overnight with polyethyleneglycol (PEG) before filtering through a 0.45 μm filter and finally transduced into ARPE-19 cells for 48 hours. The cells were then subjected to puromycin (1.0 μg/mL) selection for 10 days for the identification of resistant colonies.

Flow cytometry for Apoptosis

Cellular apoptosis was investigated using the FITC Annexin V/ PI kit and following the recommended protocol (BioLegend Inc., San Diego, USA). Briefly, 1×10^6 cells from different treatments were trypsinized, rinsed twice with cold cell staining Buffer, and suspended in 100 μl of binding buffer. Subsequently, 5 μl Annexin V-FITC was added and incubated for 10 min in dark condition at RT, followed by the addition of

10 µl PI for 5 min before flow cytometry (BD FACSVia Flow Cytometer, BD Biosciences, USA).

Autophagy flux assessment by GFP-LC3 puncta and GFP-LC3 cleavage assay

Cells expressing GFP-LC3 were generated by transfection of ARPE-19 cells grown in 6 well-plates and reaching 80% confluency with 2.5 µg pEGFP-LC3 plasmids (Addgene plasmid # 24920) using Lipofectamine 3000 (Invitrogen) for 24 h. After the desired treatments, there was autophagy flux assessment by immunoblotting for free GFP levels in a cell lysate or live-fluorescence cell imaging for GFP-LC3 puncta in the cells. For quantification of GFP-LC3 puncta, treated cells were rinsed with PBS and incubated with serum-free medium for fluorescence imaging using an inverted confocal microscope (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam) and a 40X objective. GFP-LC3 puncta were quantified from triplicates by counting a total of 30 cells as previously reported (Ni et al., 2011).

Treatment, protein extraction, and immunoblotting

After treatment of cells with trehalose, HQ (hydroquinone, Sigma-Aldrich, H9003), MG132, CQ (Chloroquine diphosphate salt, C6628, Sigma-Aldrich), NH4Cl (A9434, Sigma-Aldrich), vehicle, or a combination of these as designated, cells were trypsinized (0.25% trypsin), pelleted, and washed twice with PBS before protein or RNA extraction. Proteins were extracted using ice-cold 1X RIPA lysis buffer [0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA (Millipore, Temecula, CA)] supplemented with protease and phosphatase inhibitor tablets (Roche Applied Science, Indianapolis, IN). Samples were sonicated for 1 h on ice at 4^0C, followed by centrifugation at 18,000 g for 30 min at 4 ^{0}C and collection of

the supernatant. Bradford assay was performed to determine the protein concentration of samples and the absorbances read at 595nm wavelength on a microplate reader (Ao, Azure Biosystems Inc., Dublin, USA).

An equal amount of denatured proteins (total protein of 30 µg) of samples was loaded onto a gel for SDS-PAGE electrophoresis (10% SDS-PAGE gels). Proteins were electrotransferred from gel to an Immobilon-FL PVDF membrane (Millipore) for 2 h at 250 mA. The membrane was then blocked using 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Bio-Rad Laboratories) for 1 h at room temperature. Primary antibody incubation with anti-TFEB (D2O7D, Cell Signaling Technology, 1:500), anti-LC3 (NB100-2220, Novus Biologicals, dilution 1:1000), anti-p62 (2C11, Novus Biologicals, dilution 1:2000), anti-NRF2 (EP1808Y, Abcam, dilution 1:1000), anti-LAMP2 (sc-18822, Santa Cruz Biotechnology, dilution 1:2000), anti-cathepsin D (sc-377299, Santa Cruz Biotechnology, dilution 1:500), anti-HSC 70 (sc-7298, Santa Cruz Biotechnology, dilution 1:1000), or anti-GFP (sc-9996, Santa Cruz Biotechnology, dilution 1:1000) was performed. The membrane was washed three times followed by incubation with HRP-conjugated secondary antibodies (anti-mouse IgG (H+L), A16066, or anti-rabbit IgG (H+L), A16110; dilution 1:2000) (Thermo Fisher Scientific). After washing, mixed enhanced luminol-based chemiluminescent (ECL) substrate solution was incubated with the membrane, and immunoreactive bands imaged using the Azure c600 imaging system. (Azure Biosystems; Dublin, CA). Quantification of bands was performed using ImageJ analysis software. Protein expressions were normalized to GAPDH (AM4300, anti-GAPDH, dilution 1:2000, Thermo Fisher).

Isolation of RNA, RT-PCR, and qPCR

Briefly, RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's suggested protocol. cDNA was transcribed from 1 µg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stored for conventional reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative PCR (qPCR).

Quantitative PCR was performed in triplicate using a reaction mix of 2 µl cDNA template, 5 µl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), 1 µl nuclease-free water, and 1 µl of gene-specific primers (Table 1). Following denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s were run using the LightCycler®480 Instrument II (Roche Diagnostics, Mannheim, Germany). Fold changes were calculated using the change in Cycle threshold (ΔΔCT) method.

For conventional RT-PCR, the 20 µl reaction mix contained 1.5µl cDNA template, 10µl 2x Taq HS mix (R028A, Premix Taq™ DNA Polymerase Hot-Start Version), 1 µl each of forward and reverse primers (10 µM) and 6.5 µl nuclease-free water. Amplification was performed for 25 cycles of 94℃ for 30 s, 60 °C for 30 s, and 72℃ for 30 s (MJ Research PTC-200 Gradient Thermal Cycler, USA). The amplified products were loaded and analyzed by 1.5% agarose gel electrophoresis containing GelGreen nucleic acid stain (Biotium Inc., Hayward, CA, USA) and visualized under UV light (Gel Doc/ChemiDoc Imager, Azure, Dublin, CA, USA). B-actin was used as a reference for the normalization of expression of other genes.

Data Analysis

Data were analyzed using GraphPad Prism All data are presented as mean ± SD. Unpaired t-test or one-way ANOVA followed by Dunnett's multiple comparison *post hoc* tests, were used as appropriate to determine the difference between treatments. Statistical significance was set at $p < 0.05$.

Results

Trehalose increased autophagy flux in RPE cells

Autophagosome formation is essential in autophagy degradation (Yu et al., 2018). Due to the relevance of autophagosome cargoes in autophagy, its monitoring using the marker lipidated LC3 (LC3-II), an autophagosome membrane-bound protein, provides vital information about the process. Without any obstruction of the autophagy flux, the accumulation of LC3-II correlates with the induction of autophagy (Orhon & Reggiori, 2017). Hence, to determine whether trehalose induced autophagy, the changes in LC3-II protein expression level were evaluated. Incubating ARPE-19 cells with varying doses of trehalose led to the accumulation of LC3-II dose-dependently (Fig. 5.1 A & C), indicating an increase in autophagosomes by trehalose. Also, the expression of LC3-II increased time-dependently when cells were incubated with 100 mM trehalose (Fig. 5.1 B & D).

Next, changes in the autophagy flux by trehalose in the presence of the autophagy inhibitor chloroquine (CQ) were investigated, by assessment of the endogenous LC3-II levels, the accumulation of GFP-LC3 puncta in GFP-LC3 transfected cells, and the formation of free GFP fragments due to the proteolytic cleavage of GFP-LC3. To inhibit autophagy, cells were incubated with 50 μM CQ, due to the toxicity and dose-response changes previously reported (Chen et al., 2011; Yoon et al., 2010). In the

presence of CQ, trehalose treatment increased the endogenous LC3-II expression, indicating an upregulation of autophagy flux by trehalose (Fig. 5.2 A).

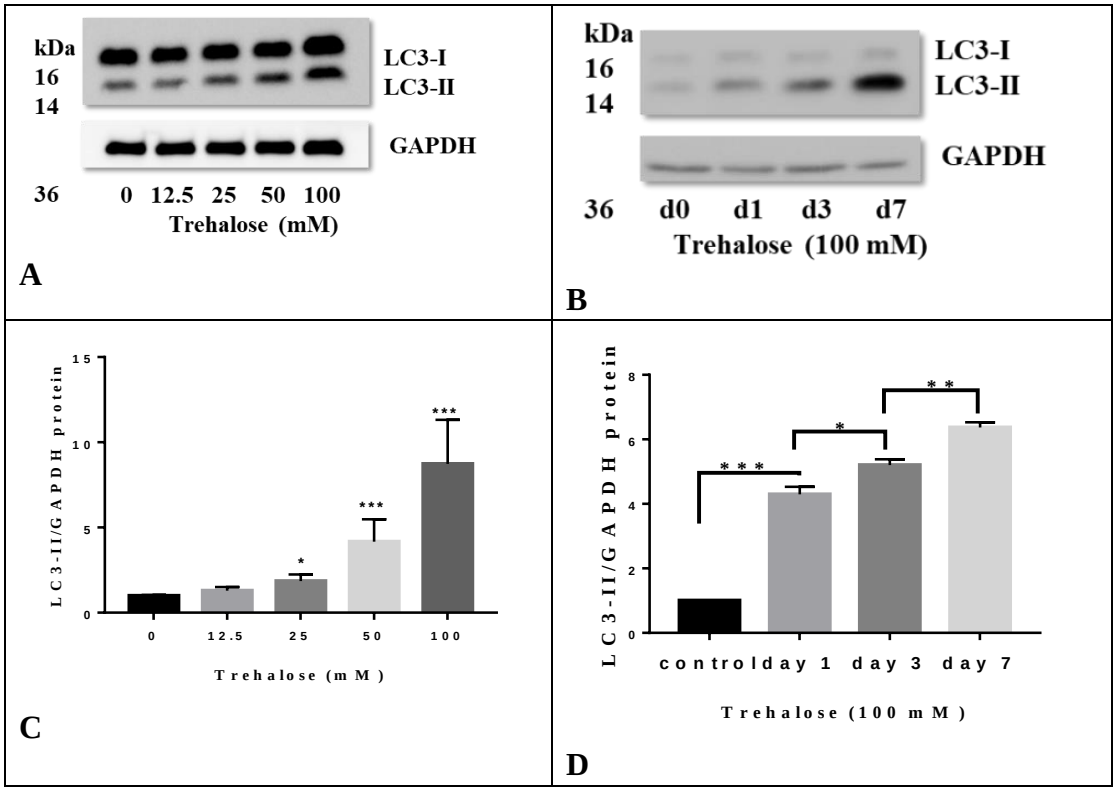


Figure 5.1: Trehalose increased autophagosomes formation in human RPE cells.

(A–D) Endogenous LC3-II expression in cell lysate from ARPE-19 cells treated as designated. Whole-cell lysate was used for western blotting with antibody for LC3-II. Protein expression level was normalized with GAPDH and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001

Also, the diffuse cytosolic staining pattern of the GFP-LC3-expressing cells became more punctate with trehalose treatment (Fig. 5.2 B), reflecting higher recruitment of GFP-LC3 to autophagosome membranes (Welter et al., 2010). The data showed that the percentage of cells with GFP-LC3 >10 puncta was increased by trehalose (p < 0.001, Fig. 5.2 C). Furthermore, analysis of the proteolytic degradation of GFP-LC3 in the transfected cells by trehalose also confirmed an upregulation of autophagy flux. There was a dose-dependent increase in free GFP level by trehalose in the presence of

CQ (Fig. 5.2 D), revealing elevation in the degradation of GFP-LC3 within autolysosomes since the LC3 portion of the fusion protein is rapidly degraded than GFP (Ni et al., 2011). Without CQ, however, free GFP level was increased when cells were treated with 50 mM trehalose but decreased with the higher trehalose dose (100 mM) (Fig. 5.2 D). When the lysosomal activity is very high, both free GFP fragments and LC3 portions are degraded together, as happens under prolonged starvation (Ni et al., 2011). This may explain why there was a decline in free GFP levels with 100 mM trehalose treatment in the absence of CQ, but in the presence of CQ, this was reversed because CQ impairs lysosomal degradation revealing the true level of autophagy flux (Ni et al., 2011). Collectively, the results corroborate increased autophagosome formation and autolysosome degradation by trehalose in RPE cells.

Autophagy induction by trehalose is not dependent on apoptosis

Cells with phagocytic functions, including RPE, liver cells, and macrophages, respond to apoptosis by inducing LC3-II-associated phagocytosis (LAP) to restore cellular homeostasis (Arandjelovic & Ravichandran, 2015; Vernon & Tang, 2013). There is, therefore, the possibility that trehalose promotes apoptosis, causing the induction of LAP, which is being confused with canonical autophagy (Martinez et al., 2011; Vernon & Tang, 2013). Also, LC3-II accumulation due to the upregulation of autophagy could be a transduction signal for apoptotic cell death (Young et al., 2012). Hence, it was investigated whether trehalose-induced LC3-II was linked to apoptosis, by studying the annexin V/PI staining pattern in trehalose-treated cells using flow cytometry. It was found that 100 mM dose of trehalose did not alter the apoptotic pathway (Fig. 5.3),

elucidating that autophagy induction by trehalose was not dependent on apoptosis and also did not promote apoptotic cell death.

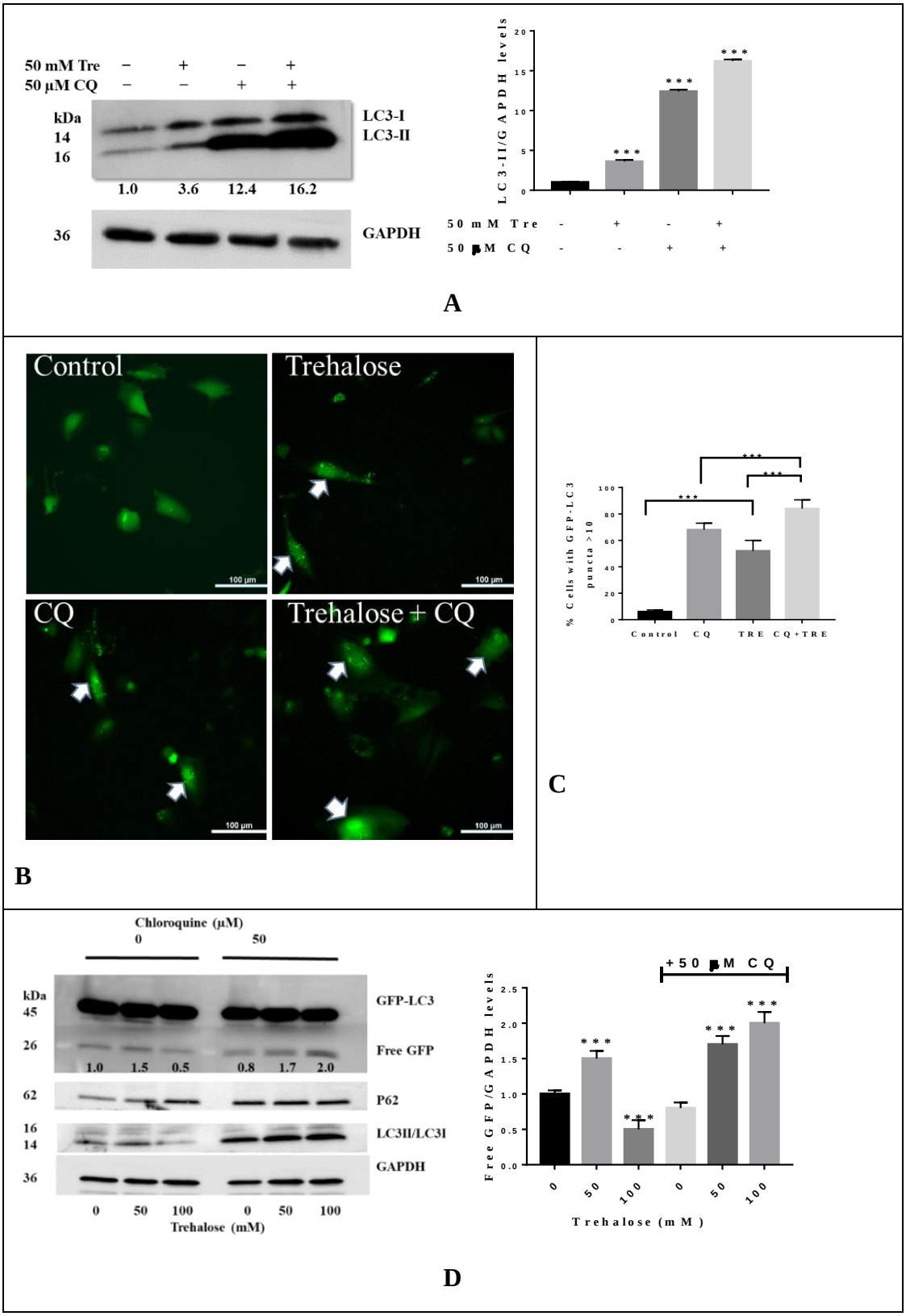


Figure 5.2: Trehalose upregulated autophagy flux in human RPE cells.

(A) Trehalose increased endogenous LC3-II levels in the presence of chloroquine (CQ) in wildtype cells. (B & C) Live-fluorescence microscopy showing percentage of GFP-LC3–expressing cells containing >10 GFP-LC3 puncta after treatment as designated. (D) Free GFP levels in cell lysate from GFP-LC3–expressing cells after treatment. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001.

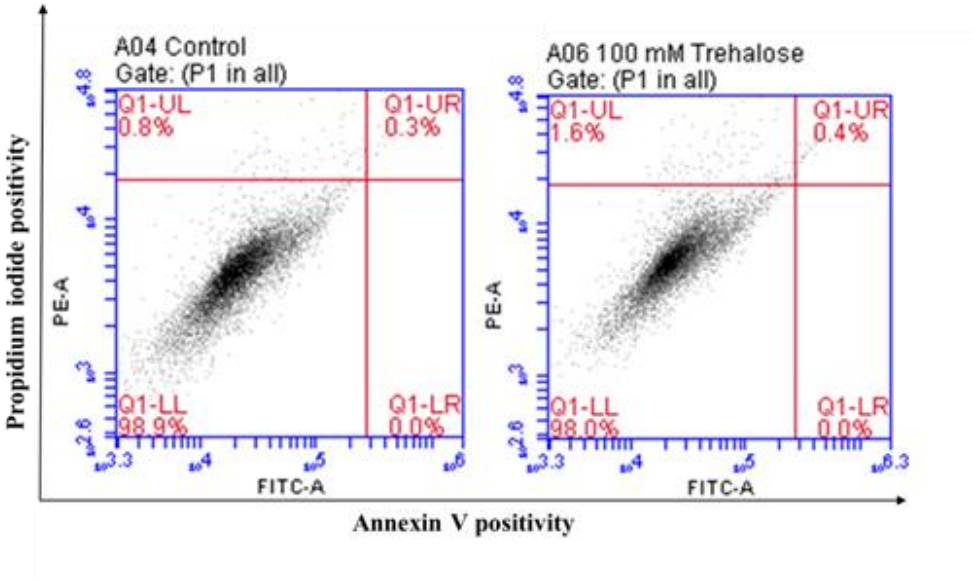


Figure 5.3: Trehalose treatment did not induce apoptosis in RPE cells.

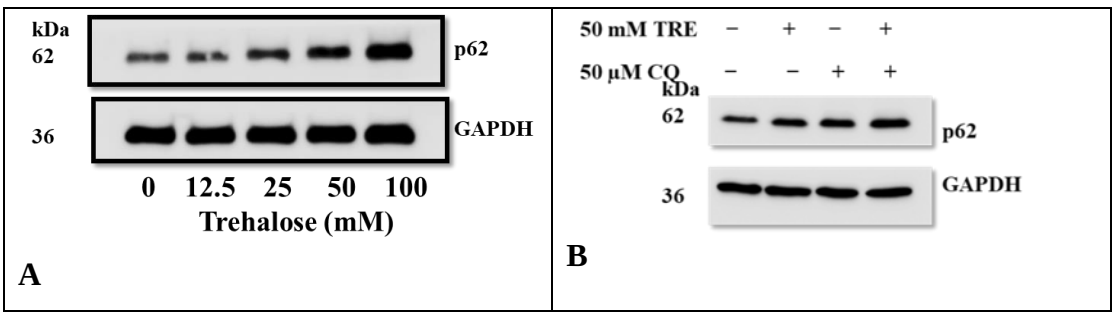
Flow cytometry results of Annexin V/PI staining in wildtype cells incubated with trehalose for 24 h. Percentage of late apoptosis and early apoptosis are shown in the upper right and lower right quadrants, respectively.

Trehalose upregulates p62 mRNA and protein expression levels and p62 turnover

The p62/SQSTM protein level may change in cells during autophagy induction (Katsuragi et al., 2015). This is because the p62 adaptor protein binds to other cytosolic autophagy substrates and become sequestered by autophagosomes, through interaction with LC3-II, which are degraded by lysosomes (Johansen & Lamark, 2011). Hence, the p62 protein level is reduced when autophagy is stimulated (Bjørkøy et al., 2009). It was observed that trehalose increased p62 protein expression dose-dependently in the RPE cells (Fig. 5.4 A & C). We investigated whether the elevated p62 protein expression

level in the cells incubated with trehalose was related to transcriptional upregulation of p62, as occurs in prolonged starvation-induced autophagy (Sahani et al., 2014). The 50 mM trehalose was subsequently used when co-treatment of cells with trehalose and other drugs was desired since we determined that it was the safest and optimal dose that did not cause any changes in cell viability and morphology. Quantitative PCR confirmed the upregulation of p62 mRNA expression in cells incubated with 50 mM trehalose compared to control (Fig. 5.4 E). Thus, the accumulation of p62 in cells treated with trehalose may be related to the upregulation of p62 at the transcriptional level, through increased synthesis of the protein.

p62 turnover by trehalose was determined to evaluate the effect of trehalose on p62 synthesis. The basal level of p62 synthesis in RPE cells was determined by incubating cells with chloroquine (CQ) to inhibit autophagy and abolish p62 degradation**.** The p62 protein level was compared in cells incubated for 24 h with 50 µM CQ alone to cells co-incubated with 50 mM trehalose in the presence of 50 µM CQ. Densitometric analysis of our western blot showed that cells co-incubated with trehalose in the presence of CQ expressed higher p62 level compared to those incubated with only CQ or trehalose (Fig. 5.4 B & D). These results support an increase in the synthesis in p62 by trehalose, as lysosomal degradation was abolished in both samples.



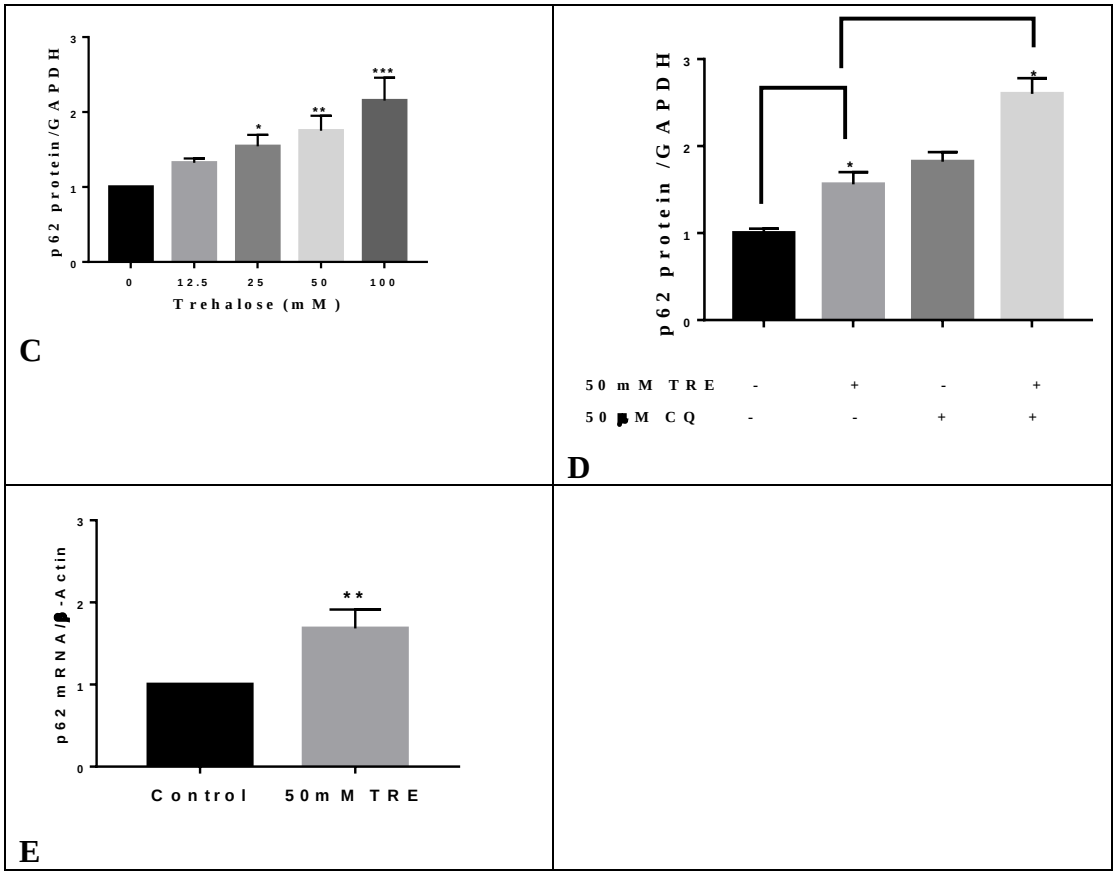


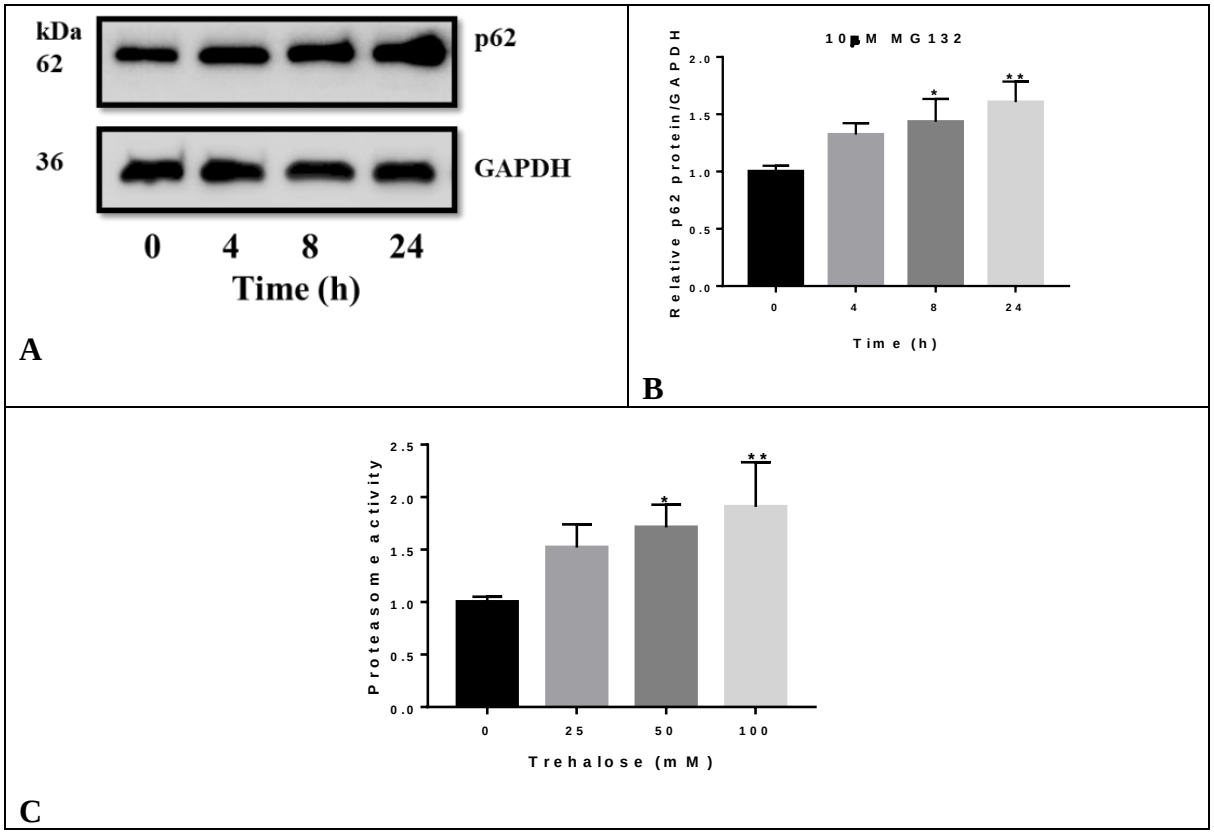
Figure 5.4: Effect of trehalose on p62 mRNA and protein expression levels and p62 turnover.

(A & E) Trehalose upregulated p62 expression at the transcription and protein levels. (A−D) Immunoblotting with p62 antibodies in whole-cell lysate from treated cells (E) RNA extraction for RT-qPCR to evaluate the gene expression level of p62. (B) Trehalose increased p62 in the presence of chloroquine (CQ). Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by the unpaired *t* test or one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

Trehalose enhanced proteasome degradation involved in downregulating p62 protein levels

The UPS also plays an important role in proteolysis and may be involved in regulating the p62 level in RPE cells. To determine the contribution of the UPS to the p62 level, cells were incubated with the proteasome inhibitor MG132. Inhibition of proteasome degradation by MG132 promoted the accumulation of p62 in the RPE cells (Fig. 5.5 A & B), indicating the regulatory role of UPS. On this premise, we evaluated the proteasome activity level of cells incubated with trehalose, to determine whether the

disaccharide impaired proteasome degradation and elevated p62 levels. On the contrary, trehalose significantly increased proteasome activity in the cells, indicating that impaired proteasome degradation had no role in the accumulation of p62 (Fig. 5.5 C). Also, we co-incubated cells with trehalose and MG132 to investigate the possible mechanism responsible for the increase in p62 protein level by trehalose. In the presence of the proteasome inhibitor MG132, trehalose still increased the p62 level (Fig. 5.5 D & E). Collectively, the evidence points to increased synthesis of p62 as the cause of the upregulation of p62 expression instead of inhibition of proteolysis by trehalose.



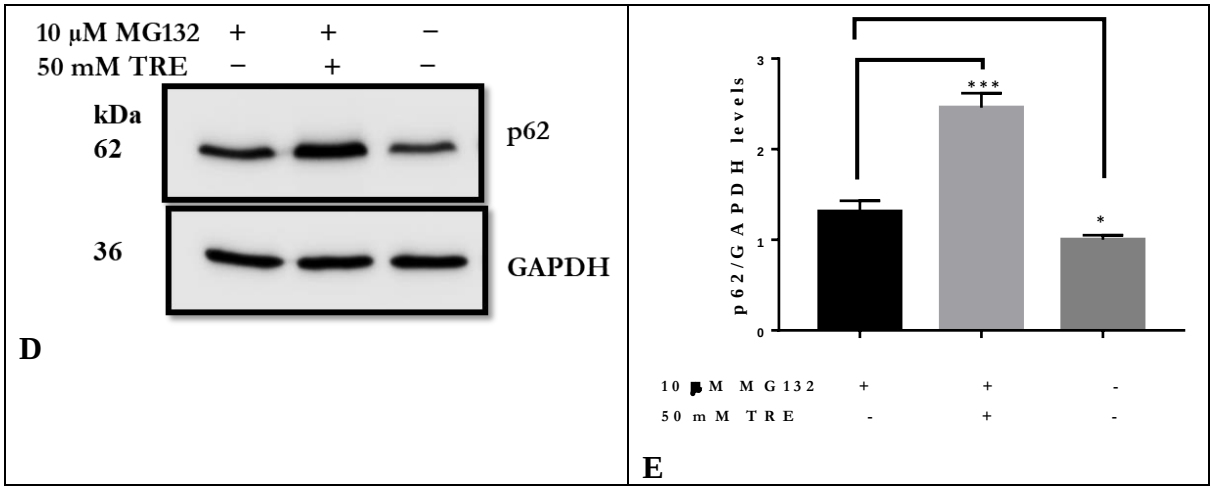


Figure 5.5: Accumulation of p62 by trehalose was not due to impaired proteasome degradation.

(A &B) Time-dependent increase in p62 protein level by MG132 in cell lysate using immunoblot. (C) Proteasome activity level in whole-cell lysates from cells (D) Trehalose increased p62 level in the presence of MG132. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.001$ vs control.

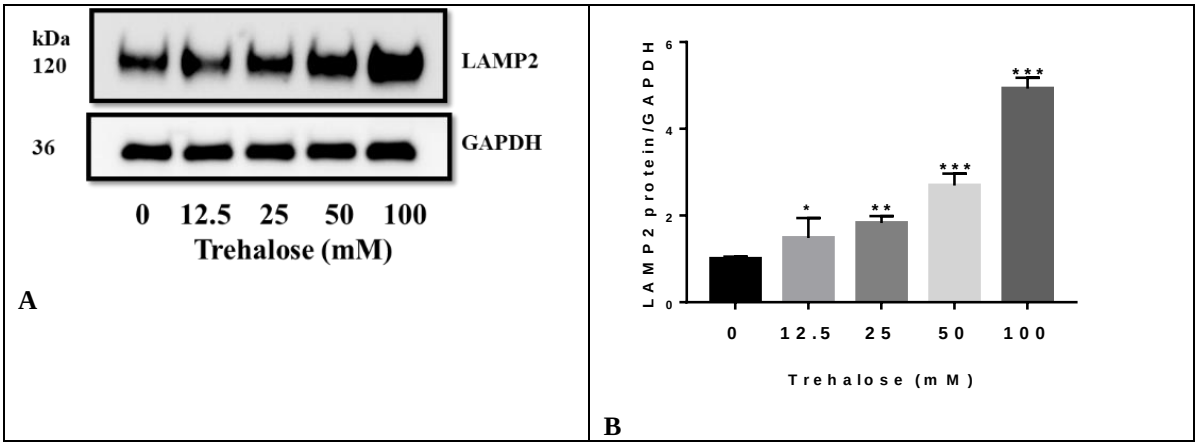
Trehalose promotes CMA and lysosomal degradation

LAMP2 is a lysosomal membrane protein that regulates the formation of autolysosomes, as it promotes the fusion of autophagosomes with lysosomes (Fortunato et al., 2009). Also, the LAMP2 expression level is the limiting factor in the degradation of substrates via CMA, a selective lysosomal degradative pathway that depends on LAMP-2A (Susmita Kaushik et al., 2011; L. Yu et al., 2018). Therefore, the effect of trehalose on autolysosome formation as well as CMA was investigated by examining the LAMP2 expression changes by western blot. Our results showed that LAMP2 expression was upregulated dose-dependently in cells incubated with trehalose (Fig. 5.6 A & B), providing evidence for an increase in autolysosome formation and CMA in the presence of the disaccharide.

Hsc 70 is another essential molecular chaperone involved in the regulation of CMA, by facilitating the specific recognition of CMA substrates in the cytosol for lysosomal uptake (Susmita Kaushik & Cuervo, 2012). The binding of Hsc70 to the KFEFQ-motif

is sufficient and necessary for lysosomal uptake of a substrate for degradation (Susmita Kaushik & Cuervo, 2012). We, therefore, determined whether any changes occurred in the level of this constitutively expressed protein by trehalose, such as found when treated with the autophagy inhibitor and neurotoxin rotenone (Sala et al., 2016). Interestingly, trehalose did not affect Hsc 70 protein expression (Fig. 5.6 C & D).

To promote autolysosome formation necessitated the upregulation of lysosomal degradation (Hundeshagen et al., 2011), the final stage in autophagy, which is completed by the hydrolytic enzymes within the lysosomal lumen. Among the several hydrolases, cathepsins are noted for the degradation of a wide range of autophagy substrates (Benes et al., 2008). Inhibition of cathepsin B and D was found to impair autolysosome degradation in fibroblasts (Tatti et al., 2013). Hence, the effect of trehalose on cathepsin D protein expression in the RPE cells was determined. The results demonstrated that trehalose upregulated the expression of cathepsin D dose-dependently (Fig. 5.6 E & F), indicating an enhancement of degradation by lysosomes. These results also support an enhancement of autolysosome formation and CMA by trehalose in the human RPE cells.



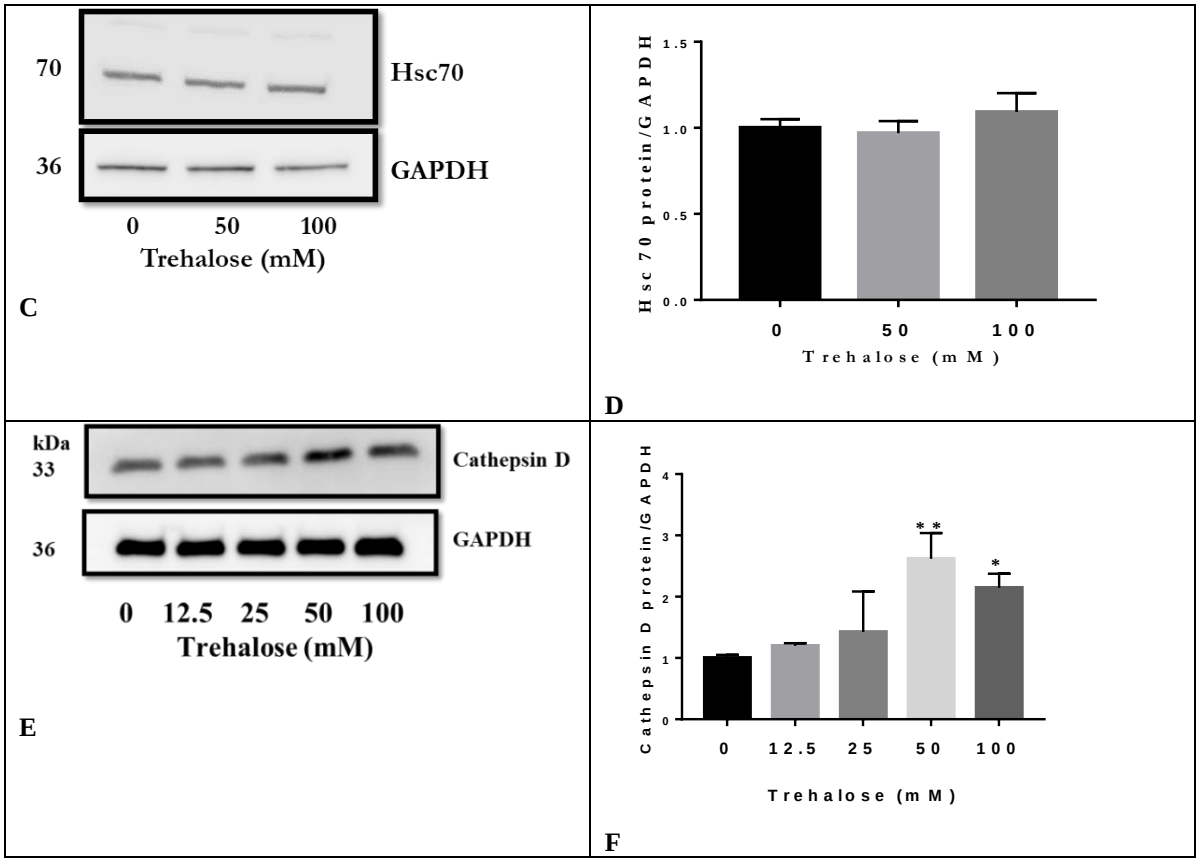


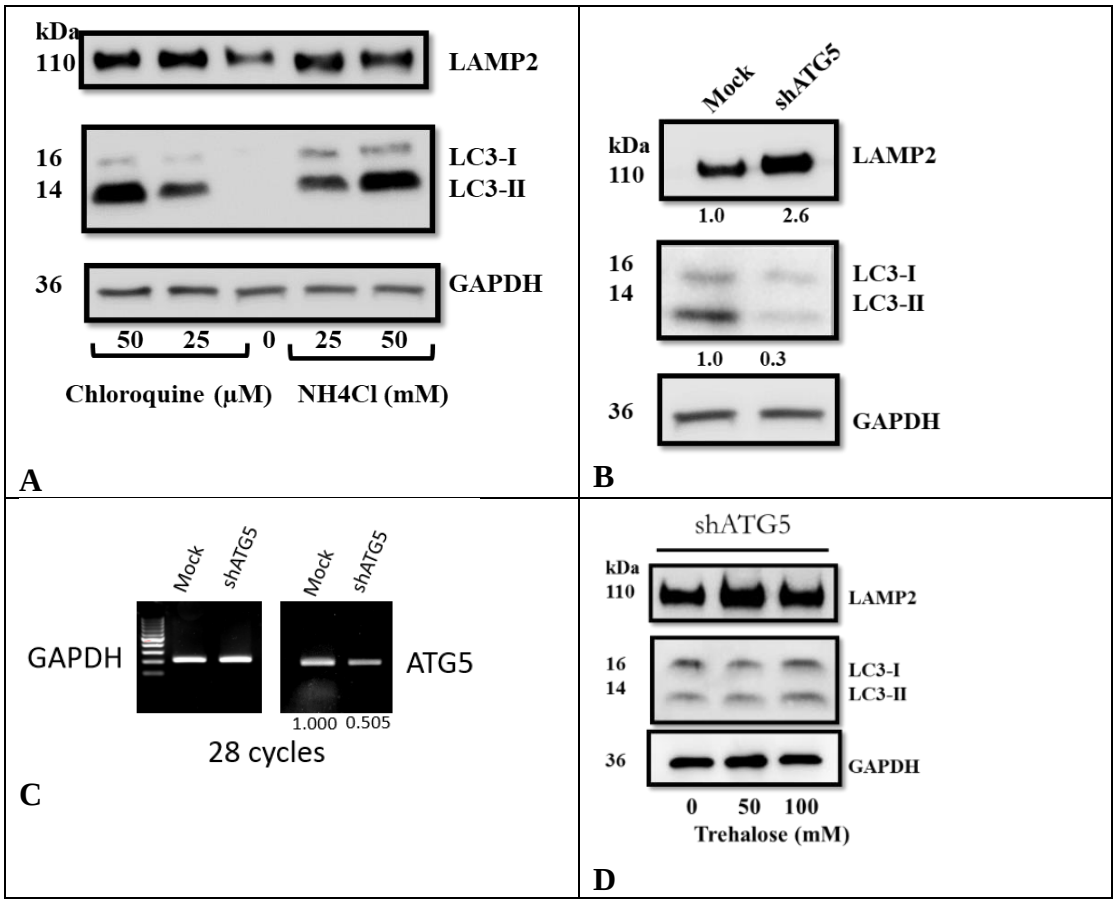
Figure 5.6: Effect of trehalose on LAMP2, Hsc 70, and cathepsin D protein levels in ARPE-19 cells.

(A-D) ARPE-19 cells were treated with different doses of trehalose for 24 h. (A & B) The whole-cell lysate was used for western blotting with appropriate antibodies. (C & D) Protein expression levels on PVDF blots were quantified by densitometry, normalized to GAPDH and expressed as a ratio of the control. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

Trehalose upregulates LAMP-2 via a macroautophagy-independent mechanism

Under certain conditions of stress, CMA may be upregulated to compensate for macroautophagy inhibition (S. Kaushik et al., 2008). We validated the existence of this kind of "crosstalk" between the two pathways in the RPE cells by inhibiting macroautophagy with two lysosomotropic agents (CQ and NH_4Cl) or transfection of cells with shRNA ATG5. Our data showed inhibition of macroautophagy with CQ or NH_4Cl (evident by the accumulation of LC3-II) upregulated the LAMP2 protein expression dose-dependently (Fig. 5.7 A), supporting CMA upregulation to compensate

for impaired macroautophagy. Similarly, LAMP2 was highly expressed in the shATG5-knockdown cells compared to wildtype cells, indicating increased CMA activity (Fig. 5.7 B). Autophagy inhibition in the shATG knockdown cells was validated by RT-PCR for ATG5 mRNA level (Fig. 5.7 C) and western blot for LC3-II level (Fig. 5.7 B). To demonstrate that trehalose upregulates CMA independent of macroautophagy, we incubated the shATG5 cells with different doses of trehalose for 24 h and examined the LAMP2 expression levels. Interestingly, trehalose increased LAMP2 in the ATG5 knockdown autophagy-defective cells without any change in LC3-II (Fig. 5.7 D & E). These data demonstrated that LAMP2 upregulation by trehalose was independent of macroautophagy.



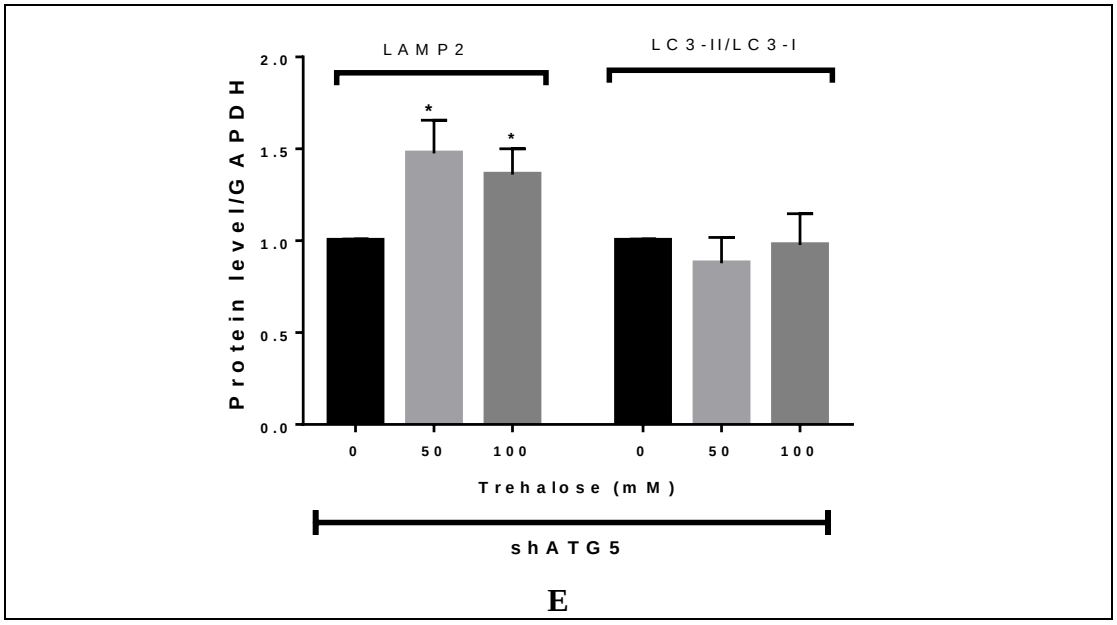


Figure 5.7: Trehalose promotes LAMP-2 expression independent of macroautophagy in human RPE cells.

(A & B) Immunoblots showing an upregulation of LAMP2 expression by autophagy inhibitors chloroquine (CQ) and NH4Cl, and shRNA ATG5 knockdown. (C) RT-PCR evaluation of ATG5 knockdown. (D) Immunoblots showing increased LAMP2 in ATG5 knockdown cells without affecting macroautophagy. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison *post hoc* tests. **p < 0.01, ***p < 0.001 vs control.

Transcriptional upregulation of TFEB and autophagy by trehalose

TFEB plays a pivotal role in the regulation of autophagy (Cortes & La Spada, 2019; Settembre et al., 2013). TFEB overexpression leads to the activation and nuclear translocation of TFEB, resulting in the upregulation of lysosomal genes and autophagy (Cortes & La Spada, 2019; Settembre et al., 2013). Also, TFEB activation has positive feedback on its own transcriptional expression, which is evident in starvation where TFEB activation and nuclear translocation, leads to the upregulation of TFEB mRNA level in mice (Settembre et al., 2013). Hence, increased TFEB mRNA and protein expression is an indication of TFEB activation and autophagy. This investigation showed that TFEB was upregulated at the transcriptional and protein levels in the

trehalose-treated cells compared to control (Fig. 5.8 A−C). Also, the mRNA levels of ATG5 and ATG7 were increased in the trehalose-treated cells compared to control (Fig. 5.8 C). These findings reveal transcriptional regulation of TFEB and autophagy by trehalose, corroborating the results of our earlier work and that of other researchers (Lotfi et al., 2018; Rusmini et al., 2019a).

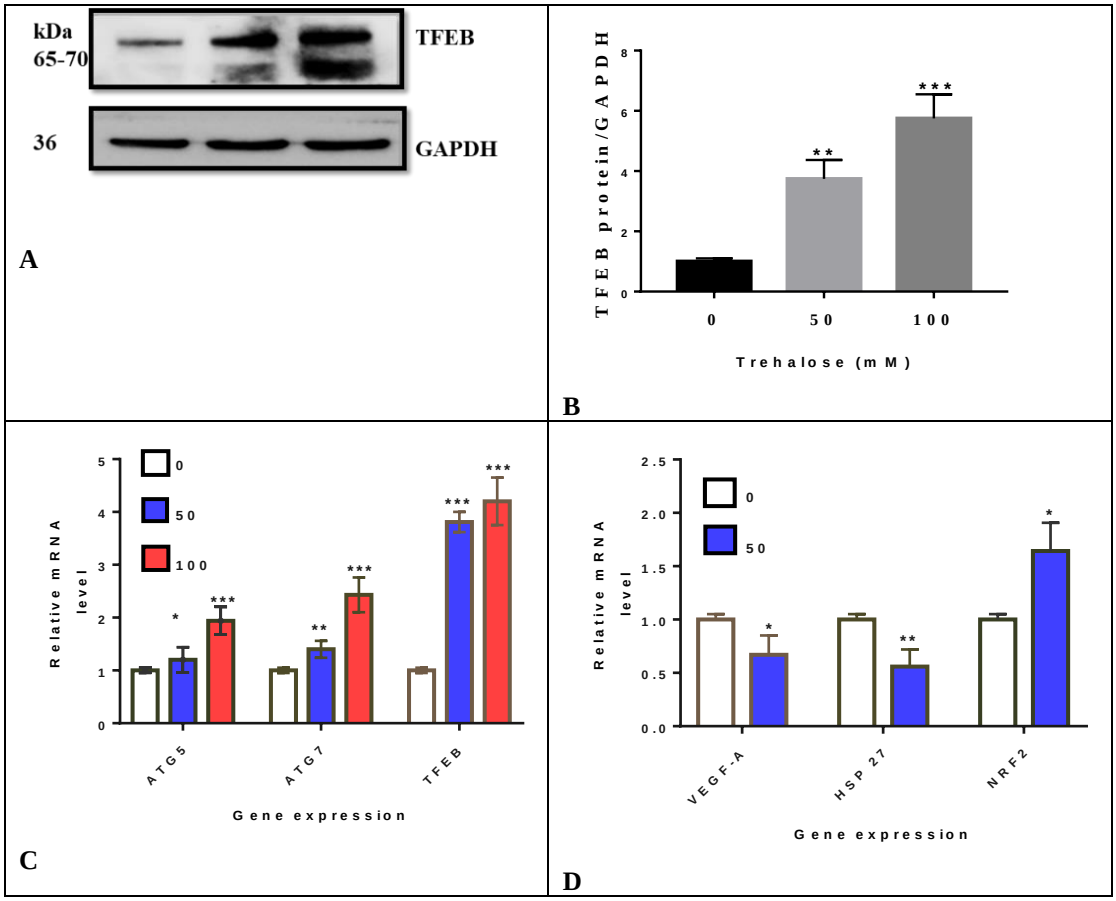


Figure 5.8: Changes in the mRNA and/or protein levels in the autophagy-lysosomal pathway and oxidative stress..

(A & B) Immunoblot showing an increase in the expression of TFEB in whole-cell lysate by trehalose. (C & D) RT-qPCR showing transcriptional changes in autophagy genes ATG5 and ATG7 and oxidative stress pathway targets, including *NRF2, HSP 27, and VEGF-A*. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using either unpaired *t* test or one-way ANOVA test, followed by Dunnett's multiple comparison *post hoc* test; *p < 0.05, **p < 0.01, ***p < 0.001.

Transcriptional regulation of NRF2, Hsp27, and VEGF-A

The effects of trehalose on the gene expression levels of key protein molecules implicated in or linked to oxidative stress and AMD were explored. These included the vascular endothelial growth factor-A (VEGF-A), heat shock protein 27 (Hsp27), and transcription factor nuclear factor erythroid 2-related factor 2 gene (*NFE2L2/NRF2)* (Klettner & Roider, 2009; Pons et al., 2010; Robledinos-Antón et al., 2019). In the retina or RPE of AMD donor eyes, the protein or mRNA levels of VEGF-A and Hsp27 are upregulated, while NRF2 is inhibited (Decanini et al., 2007; Grisanti et al., 2015; Lei Wang et al., 2014). The upregulation of individual VEGF-A isoforms disrupts retinal homeostasis, inciting the onset and progression of neovascular AMD (Grisanti et al., 2015). As NRF2 activation protects against oxidative stress its impairment promotes oxidative damage in RPE, an insult that drives AMD. Hsp27 is a molecular chaperone that is reported to regulate misfolding of proteins, actin reorganization, and key components of the apoptotic signaling pathway in the retina (Benn et al., 2002; Pons et al., 2010; Tezel & Wax, 2000), but its exact role whether protective or harmful is controversial. It was reported to contribute to RPE membrane blebbing and sub-RPE deposits in mice exposed to the cigarette smoke oxidant HQ (Pons et al., 2010). Cells treated with trehalose downregulated the mRNA levels of VEGF-A and Hsp27 and upregulated the NRF2 antioxidant transcription factor relative to control (Fig. 5.8 D). These results suggested that trehalose might modulate oxidative stress in the human RPE cells.

Autophagy induction by trehalose was not associated with oxidative stress or cell death

Under conditions of stress or cell death, autophagy is upregulated for cytoprotection (Vakifahmetoglu-Norberg et al., 2015). Hence, investigating whether an autophagy inducer increased reactive oxygen species (ROS) production and its cytotoxicity is relevant to inform on the therapeutic role of that agent. Trypan blue dye exclusion assay revealed no changes in the viability of human RPE cells incubated with doses of trehalose up to 200 mM for 24 h (Fig. 5.9 A), indicating that trehalose has a wide non-toxic dose range for harnessing its autophagy effect. Also, the CM-H2DCFDA assay for intracellular ROS measurement showed that the cells incubated with trehalose for 24 h had significantly lower ROS levels compared to control (Fig. 5.9 B). Altogether, the results showed that autophagy induction by trehalose was independent of oxidative stress or toxicity, as the disaccharide rather possessed antioxidant properties.

Trehalose protects against hydroquinone (HQ)-induced oxidative damage

Mice fed with the cigarette smoke oxidant HQ developed sub-retinal deposits, oxidative damage of the RPE, and retinal degeneration (Bertram, Baglole, Phipps, & Libby, 2009; Masashi Fujihara et al., 2008). These findings implicated HQ as a risk factor for developing AMD in cigarette smokers. We evaluated the cytoprotective effect of trehalose against HQ-induced toxicity in RPE cells using assays for protein carbonyl and cell viability. Protein carbonyl is a stable and reliable marker that directly correlates with oxidative stress levels (Dalle-Donne et al., 2003). When cells were exposed to 50 µM HQ for 2 h without trehalose pretreatment, cells showed significantly lower viability and higher protein carbonyl levels compared to control ($p < 0.001$, Fig. 5.9 C & D), indicating increased oxidative damage. However, the pretreatment of cells with

trehalose for 24 h prevented the loss of cell viability and increase in the protein carbonyls level, in a dose-dependent manner (Fig. 5.9 C & D). Strangely, it was found that doses of trehalose of 100 mM trehalose or above were not protective, despite their potent-ROS inhibition (Fig. 5.9 C). This observation also contributed to the use of a lower dose of trehalose (50 mM) throughout the experiments. It is possible that the marked reduction in ROS levels, by higher doses of trehalose, disrupts redox signaling pathways. While ROS were regarded as harmful, it is emerging that they are relevant signal transducers and involved in regulation of several cellular processes (Finkel, 2011). Perhaps, also the upregulation of autophagy contributed to the loss of viability, as beyond certain limits autophagy could trigger apoptosis (Mukhopadhyay et al., 2014).

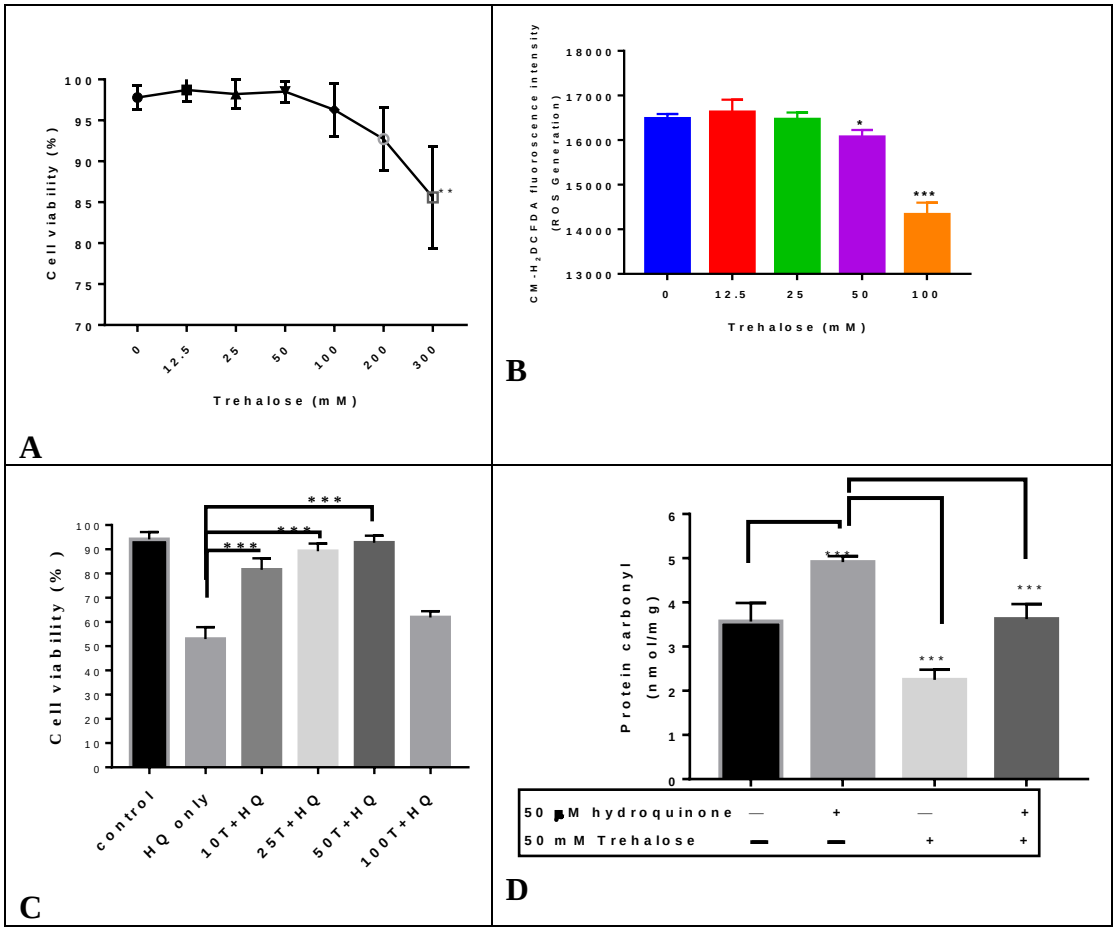


Figure 5.9: Cytoprotection of trehalose against oxidative damage by hydroquinone (HQ) in ARPE-19 cells.

(A) Trypan blue assay reveals that trehalose is non-toxic over a wide dose-range. (B) Trehalose decreased basal level of reactive oxygen species (ROS) assessed using CM-H_2DCFDA assay (C & D) Trehalose pretreatment improves the viability of cells and lowered the protein carbonyl levels in cells exposed to HQ. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett's multiple comparison *post hoc* test; $*p < 0.05$, $***p < 0.001$.

Trehalose's cytoprotection against HQ-induced oxidative damage was dependent on autophagy induction

To elucidate the mechanisms behind the cytoprotection of trehalose against HQ, we studied differences in the protein expression of LC3-II, p62 and NRF2 in cells treated with 50 mM trehalose or vehicle before incubation with HQ. Cells incubated with 50 µM HQ marginally increased LC3-II ($p = 0.04$) and decreased p62 and NRF2 protein levels (Fig. 5.10 A-C, $p < 0.001$), suggesting impairment of the NRF2 antioxidant pathway while autophagy was activated (Silva-Islas & Maldonado, 2018). Trehalose treatment upregulated the expression of LC3-II, p62, and NRF2 in cells, supporting the activation of both autophagy and NRF2 pathway (Fig. 5.10 A-C). Trehalose pretreatment, however, did not prevent the depletion of NRF2 in cells following HQ exposure (Fig. 5.10 A & B), but the protein levels of LC3-II and p62 were upregulated (Fig. 5.10 C & D, $p < 0.001$). These results linked the cytoprotection of trehalose to the upregulation of autophagy and/or p62. To confirm the role of autophagy activation, the cytoprotection of trehalose-pretreatment against HQ in the shRNA ATG5 cells was also explored. The viability of shRNA ATG5 cells pretreated with trehalose was lowered to a level similar to that in the untreated cells (Fig. 5.10 E), demonstrating the loss of cytoprotection by trehalose in the autophagy impaired cells. Our data, therefore, support autophagy activation by trehalose in the RPE cells to protect against HQ.

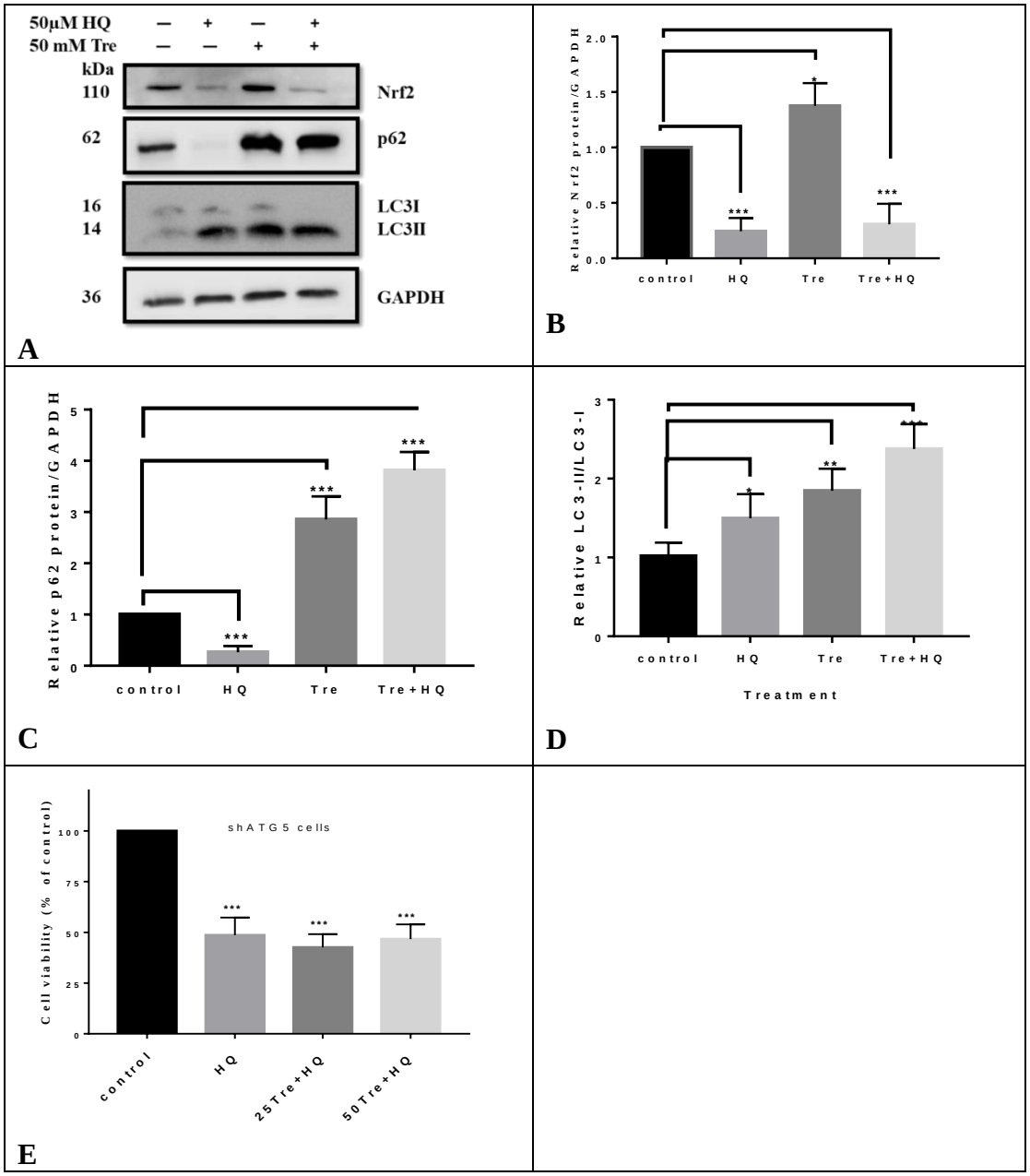


Figure 5.10: Autophagy upregulation by trehalose protects against hydroquinone (HQ)-induced oxidative stress in human RPE-19 cells.

(A-D) Immunoblots for changes in protein expression of LC3-II, p62, and NRF2 in cells preincubated with trehalose or vehicle before HQ exposure. (A-D) (E) Trypan blue assay showing loss of protection against HQ in shRNA ATG5 cells pretreated with trehalose. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett's *post hoc* test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Discussion

The retinal pigment epithelium (RPE) is an essential monolayer of pigmented cells of the retina, whose functions include the recycling of rhodopsin chromophore, nourishment of the photoreceptors, phagocytosis of photoreceptor outer segment, and formation of the retinal-blood barrier (Pavan & Dalpiaz, 2018; Strauß, 2016). The RPE cells in healthy adult eyes, similar to other neurons in the human body, are postmitotic in situ (M. Chen et al., 2016), and, hence, maintenance of RPE homeostasis is crucial for effective retinal function. In AMD and some other retinal degenerative conditions, the RPE is the earliest targeted site of damage, particularly by oxidative stress (Pavan & Dalpiaz, 2018). Therefore, interventions protective against oxidative damage in the RPE may have therapeutic relevance in the management of retinal diseases. Data from this study showed that autophagy induction by trehalose effectively protected human RPE cells against HQ-induced oxidative stress. This has important clinical implications, since finding a treatment modality that inhibits oxidative damage in human RPE cells by cigarette smoke oxidants could potentially prevent the development of AMD. Cigarette smoking, an important risk factor in AMD, causes oxidative damage to the RPE, and HQ in the smoke/tar was implicated as a major oxidizing agent (Espinosa-Heidmann et al., 2006; Masashi Fujihara et al., 2008; Tsujinaka et al., 2015).

The current study investigated the autophagy-stimulating effect of trehalose. Since autophagy is dynamic both autophagy specific markers and autophagy flux were examined to determine the effect of trehalose on each stage of the autophagy-lysosome pathway. The autophagy markers and autophagy flux were upregulated by the disaccharide in the human RPE cell line. Evidence of autophagy flux upregulation was

demonstrated by the increase in both LC3-II as well as free GFP levels in the presence of CQ in the trehalose-treated cells.

TFEB overexpression and autophagy upregulation by trehalose found in this study are consistent with recent studies investigating the effect of trehalose *in vivo* in mice and primary cells (Lotfi et al., 2018; Rusmini et al., 2019b). Thus, the autophagy-inducing effect of trehalose is not limited by cell type.

Both previous and the current studies found that trehalose increased p62, which could be suggestive of autophagy inhibition (Bjørkøy et al., 2009). The accumulation of p62 by trehalose, theoretically, could occur under two conditions; 1) inhibition of lysosomal degradation and 2) upregulation of p62 synthesis. This study demonstrated that trehalose upregulated LAMP-2 and cathepsin D, indicators of enhanced autophagosome-lysosome fusion and lysosomal degradation in cells, respectively (Susmita Kaushik & Cuervo, 2018; Tatti et al., 2013). In addition, it was shown that the accumulation of p62 was correlated with transcriptional upregulation of the p62 gene, explaining the possibility of increased p62 protein synthesis by trehalose. It is important to note that p62 accumulation under autophagy is not an isolated case for trehalose, as upregulation of p62 at the transcription level has been observed in starvation-induced autophagy (Sahani, Itakura, and Mizushima 2014). The finding of potent autophagy induction in human RPE cells by trehalose will be of great interest because of the recent insight into the role of autophagy in AMD and other retinal degenerative diseases (Ferrington et al., 2016; Moreno et al., 2018).

The results showcase trehalose as a unique autophagy inducer due to its dual stimulation of macroautophagy and CMA, and protection against oxidative stress associated with NRF2 inhibition in human RPE cells. The cytoprotective role of autophagy against

oxidative stress is being studied to facilitate the control of this process. Macroautophagy and CMA perform different lysosomal degradation roles, with the latter being more selective compared to the former (Susmita Kaushik & Cuervo, 2018). Although the two mechanisms may compensate for each other when there is a compromise in one, by way of upregulating the functional autophagy pathway, the activation of both autophagy pathways is needed for effective cellular resistance against oxidative stress (Kiffin et al. 2004). While this study directly evaluated the role of macroautophagy, the involvement of CMA could not be overlooked as trehalose efficiently upregulated LAMP2. The upregulation of the CMA receptor LAMP-2A in breast cancer cells was found to protect cells from oxidative damage and reduces oxidative modification of cellular proteins (Saha, 2012). It is, therefore, clear that the dual stimulation of both macroautophagy and CMA may be the reason for the efficacy of trehalose in dealing with oxidative stress irrespective of the impairment of NRF2 in the RPE cells.

While NRF2 activation directly inhibits the accumulation of ROS, autophagy ameliorates oxidative damage by being involved in the repair of damage caused by the ROS-induced cell injury. This occurs by the removal of damaged cytosolic molecules and organelles, including proteins, lipids, and damaged mitochondria, and recycling the nutrients for synthesis of new molecules and organelles (Giordano et al., 2013). For instance, autophagy has been shown to aid in DNA damage response through its regulation of p62 levels that could otherwise inhibit the DNA repair process (Y. Wang, Zhu, and Zhao 2017). Thus, while the role of autophagy in maintaining protein homeostasis, known as proteostasis, is essential under normal conditions (Ferrington, Sinha, and Kaarniranta 2016), it is even more beneficial under oxidative stress due to impairment of the proteasome degradation that accompanies oxidative stress in the RPE

(Fernandes et al. 2008). Upregulating autophagy can alleviate the burden in the RPE for proteolytic degradation, which is higher under oxidative stress and, therefore, may help restore proteostasis.

The impairment of NRF2 under oxidative stress-related conditions such as aging, cigarette smoking, and AMD has been reported (Cano et al., 2010; Sachdeva et al., 2014; L. Wang et al., 2014a). It was found that trehalose also promotes NRF2 expression evident by the increase of NRF2 transcription factor and protein levels and reduction of basal ROS level in the treated cells. However, NRF2 activation by trehalose did not occur in the presence of the cigarette oxidant HQ. This finding is worth noting as it suggests that strategies to inhibit oxidative stress through the activation of the NRF2 antioxidant pathway might be ineffective. This suggests the importance of targeting the autophagy pathway in the RPE cells under oxidative stress conditions. The dysregulation of the autophagy mechanism was found to be associated with susceptibility to oxidative stress and AMD (Mitter et al., 2014), an indication that autophagy promotes cell survival under oxidative stress. Although trehalose showed cytoprotection against HQ, at higher concentration of trehalose was not protective against HQ. This may be related to autophagy-induced cell death occurring due to overactivation as has been reported in some conditions (Baehrecke, 2005; Fulda, 2012; Levine & Yuan, 2005). This finding calls for exercising caution on the dose of trehalose to be used if cytoprotection against oxidative damage is desired.

The cytoprotection of trehalose against oxidative stress may be mediated by diverse biomolecular signaling pathways, including autophagy, NRF2 overexpression,

molecular chaperone Hsp 27, and VEGF, as reported here and in other studies (Hosseinpour-Moghaddam, Caraglia, and Sahebkar 2018; Khalifeh, Barreto, and Sahebkar 2019). This study, however, delineated autophagy induction by trehalose as the cytoprotective mechanism against HQ-induced toxicity, by showing that NRF2 depletion still occurred in the trehalose-pretreated cells protected from HQ damage. Furthermore, it was shown that the inhibition of autophagy, through shATG5 knockdown, resulted in almost complete loss of the protection of trehalose against HQ. Due to the vital role of NRF2 in the transcriptional regulation of antioxidant genes and enzymes to reduce ROS levels, the cytoprotection of trehalose against HQ-induced toxicity reveals autophagy as a powerful redox signaling regulator capable of compensating for oxidative stress associated with the dysregulation of NRF2. Extra measures were undertaken to eliminate potential confounders in the cytoprotection against HQ, due to a possible redox reaction could occur between trehalose and HQ. To avoid this, cells were washed three times to remove trehalose before incubation with HQ.

The autophagy machinery functions as an integrated cellular stress response, dictating the fate of cells - whether cells survive or die- depending on factors including the stress level (Fulda 2012; Tonelli, Chio, and Tuveson 2018). It was found that when cells were incubated with higher doses of trehalose, its cytoprotection against HQ-induced toxicity was diminished or lost, although higher doses of trehalose induced greater levels of autophagy and higher ROS inhibition. The loss of protection may be due to over-stimulation of autophagy resulting in apoptotic death of cells, as occurs under certain conditions such as prolonged starvation and oxidative stress (Cooper, 2018; Mukhopadhyay et al., 2014; Sadasivan et al., 2006). Thus, there may be a limit for

autophagy induction to be protective in cells, hence determining the optimal dose of trehalose for RPE protection and the management of AMD will be crucial.

Conclusion

In conclusion, this study validates trehalose as a potent TFEB-mediated autophagy inducer in human RPE cells that is cytoprotective in an *in vitro* AMD condition mimicked using hydroquinone. Studies to ascertain the neuroprotection of trehalose in alleviating animal models of AMD and retinal diseases associated with impaired NRF2 antioxidant defense are, therefore, warranted.

CHAPTER VI

Trehalose improves TFEB-mediated autophagy and cone photoreceptor function decline associated with aging in BALB/c mice

Introduction

Pathophysiology of AMD

Age-related macular degeneration (AMD) is a neurodegenerative disease affecting the central retina and contributes significantly to blindness in the aged population. AMD is characterized by the loss of central vision and abnormal retinal changes at the macular region, including damage of retinal pigment epithelium and photoreceptors (predominantly cones), the accumulation of subretinal deposits and thickening of the Bruch's membrane (Bhutto & Lutty, 2012; Kinnunen et al., 2012). In addition, the loss of choriocapillaris and decline in retinal micro-circulation have also been reported in AMD, and some believe these occur earlier in the development of the ocular disease (Biesemeier et al., 2014). The molecular pathways affected by the AMD disease include mitochondrial metabolism and adenosine triphosphate (ATP) production (Riazi-Esfahani et al., 2017; Terluk et al., 2015), glucose and lipid metabolism (Joyal et al., 2016), oxidative stress and inflammation (Datta et al., 2017; Jarrett & Boulton, 2012).

Similarities between AMD and normal age-related retinal changes

Aging is an important risk factor in AMD (Ardeljan & Chan, 2013; Ehrlich et al., 2008). Aged human eyes show changes in retinal function and structure similar to AMD**,** albeit to a lesser extent, and which in some individuals progress and develop into AMD (Ardeljan & Chan, 2013; Ehrlich et al., 2008). The effect of aging on AMD has been mostly studied in mice and rats, without which the significant insight made into the

pathophysiology of AMD would not have been possible (Forest et al., 2015; Pennesi et al., 2013; Ramkumar et al., 2010). It has been possible because the wild-type BALB/c and C57BL/6 mice also develop age-related retinal changes resembling AMD, to some extent (Bell et al., 2015; Gresh et al., 2003; Hoh Kam et al., 2015; Ida et al., 2004; Samuel et al., 2011; Yekai Wang et al., 2018). Toxic deposits of lipofuscin and amyloid β have been found to accumulate in the retina of C57BL/6 contributing to oxidative damage of the RPE, chronic inflammation and the loss of photoreceptors (Hoh Kam et al., 2015; H. Xu et al., 2008). Also, there is evidence of changes in the size of retinal ganglion cells (RGC), which interferes with the retinal circuitry and the transmission of visual signals to the brain (M. A. Samuel et al., 2011). In addition, reports have demonstrated the loss of choriocapillaris and a decline in mitochondrial membrane potential and metabolism, ATP production, and glucose metabolism in the eyes of older mice (Ida et al., 2004; Yekai Wang et al., 2018). Due to the similarities between the normal age-related retinal structure and functional changes in rodents and the AMD disease, rodents offer an adequate platform for investigating promising therapeutic interventions for AMD (Gresh et al., 2003; Hoh Kam et al., 2015; Sivapathasuntharam et al., 2017).

Aging, autophagy, and AMD

Autophagy is a catabolic lysosomal degradation of cytosolic biomolecules and organelles, getting rid of the toxic intracellular substances that could harm cells and providing them back with essential nutrients for the synthesis of new molecules for cell survival (Boya et al., 2013; Rubinsztein et al., 2011). Substrates targeted for autophagy degradation include damaged proteins, nucleic acids, lipids, mitochondria and other organelles (Filomeni et al., 2015). The accumulation of these cytoplasmic substances

is toxic to neurons and has been implicated in AMD. Autophagy upregulation, therefore, is crucial in modulating neuronal homeostasis and prevention of neurodegeneration (Filomeni et al., 2015; Metaxakis et al., 2018). The problem, however, is that autophagy may be downregulated with aging, interfering with retinal homeostasis (Lionaki et al., 2013; Metaxakis et al., 2018; Rubinsztein et al., 2011).

The disaccharide trehalose, known for promoting TFEB expression and autophagy, was found to have therapeutic effects in the treatment of different models of human neurodegenerative diseases (Emanuele, 2014; Hosseinpour-Moghaddam et al., 2018; Lotfi et al., 2018; Schaeffer et al., 2012). Earlier, we found that trehalose upregulated both macroautophagy and chaperone-mediated autophagy in human retinal pigment epithelial (RPE) cells, protecting cells against oxidative damage under conditions of NRF2 inhibition. To highlight the role of autophagy in AMD, the retinal changes in autophagy and oxidative stress with age in BALB/c mice were studied, followed by an assessment of the neuroprotection of trehalose against the retinal degeneration due to aging. Our data revealed the following: 1) there was an inhibition of TFEB expression and autophagy, and increased oxidative stress in the retina of older mice with degenerative changes; 2) the oral intake of trehalose improved autophagy in the retina and preserved cone-mediated responses in aged mice; 3) prolonged oral intake of trehalose for 6 months did not alter the plasma glucose level of mice clinically. We believe that delaying the retinal degenerative changes in aging eyes is the first step towards the prevention of AMD. This translational study, therefore, supports the therapeutic potential of trehalose-induced autophagy as a remedy for managing AMD.

Methodology

Animal care and ethics approval

BALB/C mice aged 6-8 weeks were acquired from the Central Animal Facility (CAF), PolyU. Mice were maintained in a 12-hour light-dark cycle and fed with normal chow and water *ad libitum*. License for animal use was obtained from the Department of Health and ethics approval for the study was given by the Animal Subjects Ethics Sub-Committee (ASESC), PolyU; ASESC#: 18-19/56-SO-R-STUDENT. All procedures conducted on animals adhered to the NIH Guide for Care and Use of Laboratory Animals, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental design

Firstly, BALB/c mice aged 2 months were monitored for changes in visual function and structure with age, using ERG and OCT. Measurements were performed at different time points when the mice were 2, 4, 8 and 18 months old. Mice were sacrificed at each of the time points for the assessment of autophagy and oxidative stress levels in the retina. The outcome of this study was used to plan the second experiment to evaluate the neuroprotection of trehalose against the age-related degenerative retinal changes in the BALB/c mice.

Next, to investigate the effect of trehalose on age-related retinal changes, littermate BALB/c mice aged 2 months were randomized to receive either drinking water or 2% trehalose solution *ad libitum*. To prepare a 2% (w/v) trehalose solution, 4 g trehalose (100%, Swanson Ultra) was dissolved in 200 ml drinking water in the drinking bottle for mice and changed twice/week. The trehalose treatment started when the mice were

2 months old and continued until mice reached 10 months old. The ERG and OCT measures were recorded at baseline in the 2-month-old mice and repeated in the 8-month-old and 10-month-old mice.

Electroretinography (ERG)

The flash ERG protocol was used for the functional assessment of three retinal cell types; the rod photoreceptors, rod bipolar cells, and the cone-depolarizing bipolar cells (Kinoshita & Peachey, 2018). For the standard ERG measurements using the Ganzfeld Q450C (Roland Consult, Brandenburg, Germany), the animals were dark-adapted overnight (14-16 hrs), and all handling was performed under dim red illumination. The mice were anesthetized by IP injection of 50 mg/kg ketamine (Ketavet®, Pfizer, Berlin, Germany) and 10mg/kg xylazine (Rompun® 2%, Bayer, Leverkusen, Germany). Also, mice received topical eye drops of 2.5% phenylephrine hydrochloride to dilate pupils, and 0.5% proparacaine hydrochloride to anesthetize cornea. The pedal reflex was evaluated to determine that the mouse was adequately anesthetized and ready for ERG measurement.

To measure the ERG, a mouse was placed onto the warming platform (37°C). The ground needle electrode was inserted subcutaneously at the base of the tail, the reference needle electrode was positioned medially between the ears and the two active ring electrodes were placed onto a wetted corneal surface of eyes. Following the observation of acceptably low impedance readings and adaptation for five minutes, the ERG recording began based on the protocol described below. After the measurement, mice were transferred into cages on a warming blanket (37°C) and monitored until they wake up.

For the dark-adapted stimulus series, stimuli were presented to mouse eyes in order of increasing flash strength within a Ganzfeld, a circular sphere which results in a homogeneous stimulus flash. A series of 6 flash levels were used ranging from −3.6 log cd s/m2 to 1.47 log cd s/m2. Signal averaging was used to improve the signal-to-noise ratio and there was an increase of the inter-stimulus interval between flashes as flash luminance increases (Kinoshita & Peachey, 2018). Ten successive responses were averaged at our lowest flash level and using a 10-sec inter-stimulus interval. As flash luminance increases, we decrease the number of responses averaged and increase the inter-stimulus to avoid light adaptation. The amplifier band-pass was set to 0.3 – 1,000 Hz and amplifier gain were varied according to overall response amplitude.

For the light-adapted stimulus series, mouse eyes were exposed to a 30 cd/m2 steady field of light within the Ganzfeld for 7 minutes prior to the recording of the photopic ERG. This desensitized the rods so that flash stimuli presented against the adapting field evoked only a cone-mediated response. This was followed by the superimposition of a series of 3 flash levels that ranged from 0.05 log cd s/m2 to 1.47 log cd s/m2. These were presented in order of increasing luminance and 25 successive responses to stimuli presented at 0.5 Hz were averaged. Amplifier and time base settings are the same as used for dark-adapted recordings.

SD-OCT imaging

Mice were anesthetized with a single intraperitoneal injection of ketamine (100 mg/mL), xylazine (20 mg/mL) cocktail. Also, a 1% tropicamide eye drop was instilled into the eyes to dilate the pupils, followed by the application of a gel to the corneal surface to hydrate the corneas (GenTeal liquid Novartis, East Hanover, NJ). Before and

during imaging, lubricant eye drop (Systane Ultra Alcon, FortWorth, TX) was instilled on eyes every 5 mins. SD-OCT images were taken using the Envisu R2200-HR SD-OCT device (Bioptigen, Durham, NC) with the reference arm set at 610 mm. Rectangular scans (parameters: 1000 A-scans/B-scan×100 B-scans×10 Frames/B scan) were obtained while centered on the optic nerve and all scans corresponded to a 1.8 mm×1.8 mm physical area on the tissue. The retinal layer thickness for cross-sections was averages generated, using the Nine x Nine Grid of the InVivoVue Diver (version 3.0, Bioptigen) for designated points of the retina centered on the optic nerve head.

Retinal dissection

Mice were euthanized by an overdose of ketamine/xylazine i.p. injection and cervical dislocation. Eyes were enucleated into cold PBS and dissected to separate the neural retina from pigment epithelium under a binocular Leica lens using a pair of thin tweezers. Extracted retinas were stored at -80ºC for use in western blotting or polymerase chain reaction (PCR).

Protein extraction and protein carbonyl assay

Tissue lysate from retina was extracted using ice-cold 1X RIPA lysis buffer [0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA (Millipore)] containing 1:100 protease inhibitor cocktail (Thermo Scientific). Samples were sonicated for 1 hour on ice at 4^0C, followed by centrifugation at 18000 x g for 30 mins at 4^0C. The supernatant was obtained, and protein concentrations were measured with BioRad Protein Assay (BioRad). Protein carbonyl concentration was measured in protein samples by spectrophotometry using the Oxiselect™ protein carbonyl ELISA kit (Cell Biolabs, STA-310) according to the manufacturer instructions. Briefly, tissue

lysate prepared from retinal specimens of the right eye was diluted to the specified 10µg/ml protein concentration for the assay. The protein samples were adsorbed onto a 96-well plate and incubated overnight at 4^{o}C. There was derivatization to DNP hydrazine, followed by incubation with the anti-DNP antibody for 1 hour, and HRP conjugated secondary antibody for 1 hour at RT. The enzyme reaction generated by the addition of the substrate solution was stopped within 5 minutes and the absorbance of wells measured at 450 nm wavelength using a microplate reader (Ao, Azure Biosystems Inc., Dublin, USA).

Immunoblotting

An equal amount of protein (total protein of 20 µg) extracted from cell samples of different treatments was loaded onto wells of separating gel for SDS-PAGE electrophoresis (10% SDS-PAGE gels). All samples were denatured by the addition of β-Mercaptoethanol (M6250, Sigma-Aldrich) and incubation at 90^{o}C for 5 min. Proteins were electrotransferred from gel to an Immobilon-FL PVDF membrane (Millipore) for 2 h at 250 mA using cold pack and prechilled buffer to reduce the generation of heat. After, the membrane was blocked using 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Bio-Rad Laboratories) for 1 hr at RT. Primary antibody incubation with anti-LC3 (NB100-2220, Novus Biologicals, dilution 1:1000), anti-LAMP-2 (2C11, Novus Biologicals, dilution1:1000) or anti-TFEB (EP1808Y, Abcam, dilution 1:1000) was performed for 2 hr at RT. Next, the membrane was washed 3 times for 10 mins each time followed by incubation with horseradish peroxidase HRP-conjugated secondary antibodies including anti-mouse IgG (H+ L, A16066) and anti-rabbit IgG (H+L, A16110; Thermo Fisher Scientific, dilution 1:2000). Again, the membrane was washed 3 times, 10 mins each. ECL substrate solutions were mixed and

incubated with the membrane for 5 mins, and immunoreactive bands were imaged using the Azure c600 imaging system. (Azure Biosystems; Dublin, CA). Quantification of bands was done using ImageJ analysis software. Protein expressions were normalized to B-actin (AC-15, Thermo Fisher Scientific dilution 1:2000).

Isolation of RNA, RT-qPCR

Briefly, RNA extraction was performed using Trizol (Life Technologies) and following the manufacturer's protocol. RNA samples were free from contaminants; both the A260/280 and A260/230 ratios fell within 1.9 to 2.1 (Thermo Scientific™ NanoDrop). Next, cDNA was transcribed from 1 µg DNA-free RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and stored for conventional reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative PCR (qPCR).

For quantitative PCR, a PCR of reaction volume 10 μl per well was set up in triplicate using 2 μl cDNA template, 5 μl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), 1 μl nuclease-free water, and 1 μl of gene-specific primers for ATG, ATG7, TFEB, and B-Actin. Quantitative RT–PCR was run on a LightCycler 480 System II (Roche Diagnostics). The reaction mixture was incubated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. All qRT–PCRs were run using the LightCycler 480 System II (Roche Diagnostics). Fold changes were calculated using the change in the cycle threshold ($\Delta\Delta$CT) method.

Measurement of random blood sugar and fasting blood sugar

The random blood sugar and fasting blood sugar levels were measured in blood collected from the distal tail vein of mice as previously described (K. L. Stewart, 2017). Briefly, the distal end of the tail was cleaned with warm water and the distal tip of the tail (1 mm of tail tissue) was amputated with a sharp pair of sterile surgical scissors. The tail was stroked from the base to the distal end to collect blood onto the tip of the strip inserted into the glucose meter (Accu Chek Performa) for the reading. Immediately after collecting the blood, the tail wound was cleaned with cotton immersed into alcohol. Mice were fasted from 8:30 a.m. to 2:30 p.m. prior to measurement of the fasting blood sugar, whereas the random blood sugar was taken before 12 p.m.

Statistical analysis

The GraphPad Prism 6 (GraphPad Software, Inc.) and Excel were used to analyze data and for drawing of graphs. All data are presented as mean ± SD. To determine differences between treatment groups, where only two samples were involved the Student's *t*-test was used, and where samples were more than two the one-way ANOVA, followed by Dunnett's *post hoc* test was used for multiple comparisons.

The a-wave and b-wave-amplitudes for different age groups of mice or treatment groups were compared using the two-way ANOVA with Bonferroni's correction for multiple comparisons tests. Thus, comparisons between the experimental groups were done for each single light intensity for scotopic ERG and photopic ERG. Statistical significance was set as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Aging affects visual function

Wild-type BALB/c mice have been reported to be susceptible to age-related changes in retinal function and structure (Bell et al., 2012, 2015; Gresh et al., 2003). To investigate the changes in the rod- and cone-mediated responses with age, there was repeated monitoring of the ERG waves in BALB/c mice at ages 2, 4, 8, and 18 months under scotopic and photopic conditions. Figure 1 shows a declining trend in the ERG waves with age. At 2 months postnatal, the retina of mice is fully developed, so the recorded ERG for this age was used as the baseline for comparison with the subsequent measures. Using the two-way ANOVA, the 4-month-old mice only revealed a mild decline in the scotopic a-wave (14 %, $p < 0.05$, **Fig. 6.1 B**), scotopic b-wave (10% $p < 0.05$, **Fig. 6.1 A**), and photopic b-wave (20 %, $p < 0.001$, Fig **6.1 C**), an indication of the loss of the rod-mediated and cone-mediated responses at this age. However, the 8-month and 18-month-old mice showed a prominent reduction in the scotopic and photopic waves even at low and moderate light intensities (**Fig. 6.1 A-C**). The recorded scotopic a-wave, scotopic b-wave and photopic b-wave were reduced by 44% ($p < 0.001$), 35% ($p < 0.001$) and 41% ($p < 0.001$) in the 8-month-old mice as compared to 59% ($p < 0.001$), 44% ($p < 0.001$), and 64% ($p < 0.001$) in the 18-month-old-mice, respectively. This data clearly demonstrates significant loss of the cones and rods photoreceptor function with age in BALB/c mice.

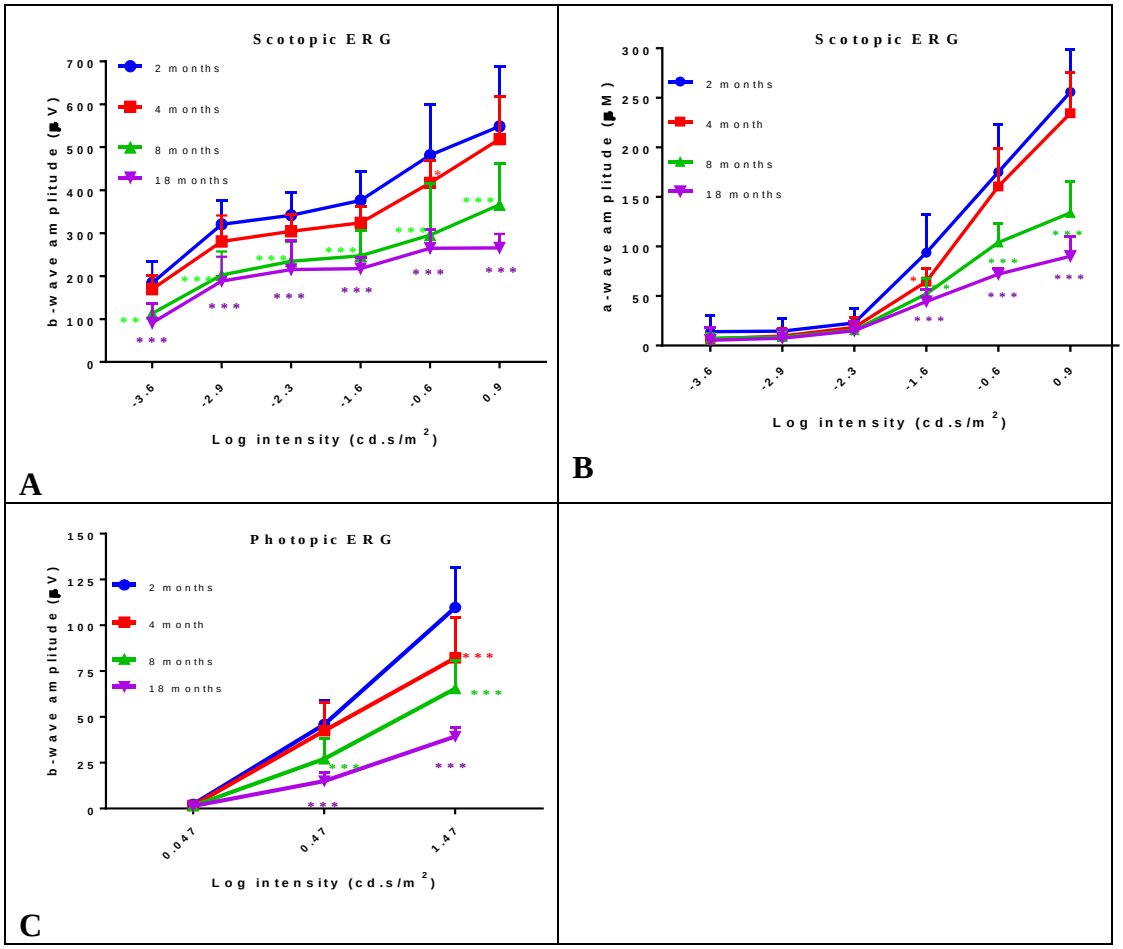


Figure 6. 1: Progressive decline in the scotopic and photopic ERG waves with age.

(A-C) Monitoring of ERG changes with age in normal wild-type BALB/C mice housed under standard lighting conditions. (A&B) Amplitude of the scotopic a-wave and b-wave was recorded in the dilated eyes of anesthetized mice dark-adapted for 14-16 hours. (C) Amplitude of the photopic b-wave was immediately recorded after at least 7 mins of light adaptation. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001 vs control.

Aging affects retinal structure

To determine whether structural changes accompanied the loss of retinal function in aging using OCT, we assessed the total retinal thickness and outer nuclear layer (ONL) longitudinally in mice at age 2, 4, 8 and 18 months. With the optic nerve head centered and using the Nine X Nine Grid of the InVivoVue Diver (version 3.0, Bioptigen), the thickness of the superior, inferior, nasal and temporal retina was measured at points 140, 280, 420 and 560 μm (**Fig. 6.2 A & B).** Our results supported a decrease in the

total retinal thickness and ONL in aging mice (**Fig. 6.2 C-E**). At the age of 2 months a total retinal thickness of 218.7 ±11.8 ±11.8 µm was recorded, declining to 206.2±13.7 µm, 195±12.3µm, and subsequently to 183.6± 6.7 µm at age 4, 8 and 18 months respectively (one-way ANOVA, F (3, 25) = 10.14, p=0.001; **Fig. 6.2 D).** Similarly, the mean ONL recorded in the 2-month-old was 64.2±7.2µm and reduced to 58.7 ±5.0µm in the 4-month-old, 54.6±6.3µm in the 8-month-old, 46.4±3.3 in the 18-month-old mice (one-way ANOVA, F (3, 25) = 10.39, p=0.001; **Fig. 6.2 E**). Collectively, these preliminary results from ERG and OCT of aged BALB/c mice support the suitability of the model for investigating the neuroprotection of interventions for retinal degeneration. In view of the significant changes in the retinal function and structure of 8-months-old mice, to overcome the long waiting time, we first tested the efficacy of our intervention after this age.

Autophagy inhibition and increased oxidative stress in the retina of aged mice

Autophagy is critical for the maintenance of neuronal homeostasis through the removal of oxidized biomolecules and organelles and the recycling of nutrients for the synthesis of new molecules (Menzies et al., 2017; Metaxakis et al., 2018). The dysregulation of autophagy is implicated in the development of age-related retinal degeneration in human eyes (Ferrington et al., 2016; Iannaccone et al., 2015). Autophagy degradation of a substrate occurs in 3 different ways; namely, macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. In mammalian cells, macroautophagy and CMA are easily monitored by the expression level of specific genes or protein markers (Patel & Cuervo, 2016; Yoshii & Mizushima, 2017).

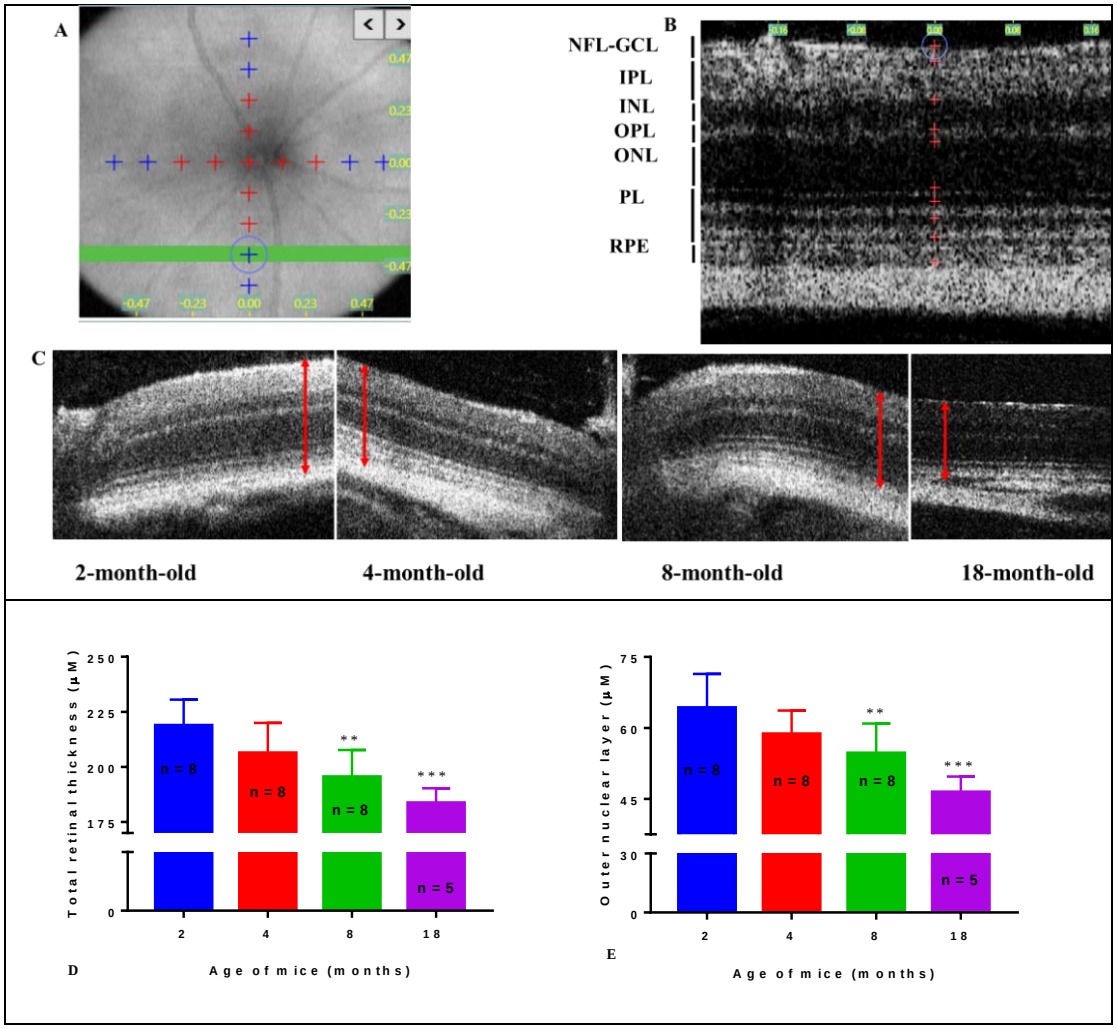


Figure 6. 2. In vivo retinal thickness measurements in young, adult and aged mice.

Mice were examined with spectral-domain optical coherence tomography (SD-OCT) at different ages. (A) Manual marking with the 9 x 9 Spider plot centered on the optic nerve head to show points (red/blue +) for thickness measurements. (B) The B scan Zoom Display showing retinal layers with markings for thickness measurement. (C) Representative OCT images of mice at age 2, 4, 8 and 18 months. nerve fiber layer (NFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); photoreceptor layer (PL); retinal pigment epithelial layer (RPE). (D & E) Statistical analysis of the total retinal thickness and ONL of mice (one-way ANOVA). Data are shown as mean+SD. Scale bar: 100 µm. *p < 0.05, **p < 0.01, ***p < 0.001 vs control.

We, therefore, compared the retinal expression of the transcription factor EB (TFEB), autophagy-related genes ATG5, ATG7, autophagosome marker LC3-II, and chaperone-mediated receptor LAMP-2A between 2-month-old mice and 8-month-old

mice. Our results showed a decline in the LC3-II and LAMP-2A with age in the retina (**Fig. 6.3** A & C), an indication of reduced macroautophagy and CMA induction (Noda & Inagaki, 2015). TFEB positively regulates lysosomal biogenesis and autophagy via stimulation of the CLEAR network of genes (Martini-Stoica et al., 2016; Napolitano & Ballabio, 2016). It was found that TFEB was decreased at both the transcription and protein levels in the retina of aged mice (**Fig**. **6.3** B-D), elucidating the mechanism behind the inhibition of autophagy with aging. Also, the ATG5 and ATG7 mRNA levels were reduced in the aged retina (**Fig. 6.3** D), supporting inhibition of the autophagosome formation process. Collectively, these results demonstrate the inhibition of autophagy in the retina of aged mice.

Protein carbonyls are formed from covalent modification of a protein either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress such as lipid peroxidation (Suzuki et al., 2010). The protein carbonyl level, therefore, provides an accurate measure of oxidative stress. To determine whether increased oxidative damage contributed to the age-related retinal degenerative changes, the protein carbonyl assay was performed to compare the protein carbonyl level in the retina of young and older mice. Our data showed that the protein carbonyl level was 2.5-folds higher in the 8-month-old mice compared to the 2-month-old mice ($p < 0.05$, **Fig. 6.3** D).

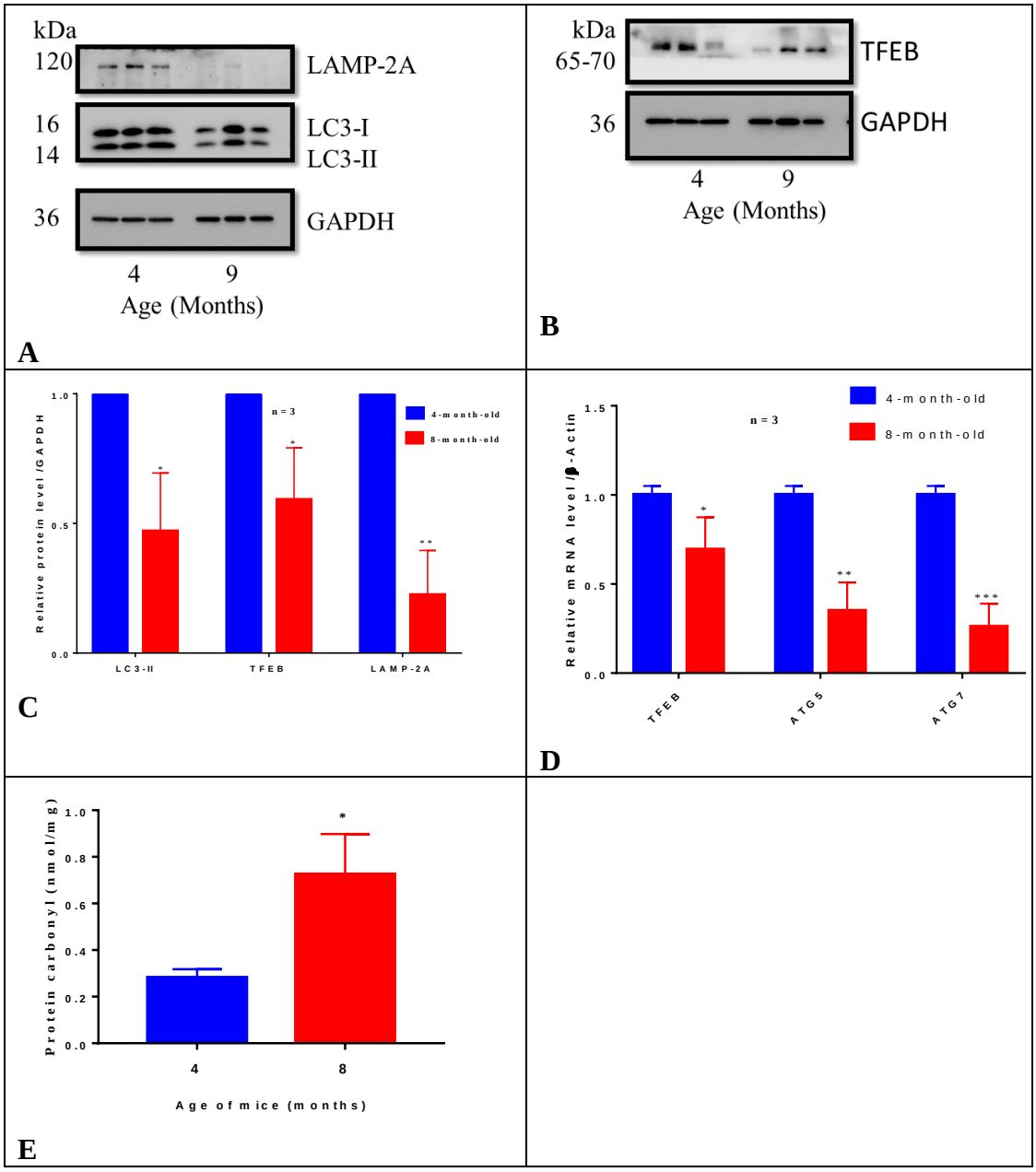


Figure 6. 3: Comparison of autophagy and protein carbonyl level in the retina of adult mice vs. aged mice with degenerative changes.

(A-D) Autophagy declines in older BALB/c mice. Mice (n=3) at the designated ages were sacrificed, eyes enucleated, and retinal tissue harvested. (A & B) Micrograph of immunoblot for tissue lysate from retinal specimens of the right eye using antibodies for LC3-II, LAMP-2A, TFEB and GAPDH. (C) Densitometric analysis of proteins was normalized with GAPDH. (D) RT-qPCR to quantify TFEB, ATG5 and ATG7 in mRNA samples extracted from retinal specimens of the left eye. The mRNA expression was normalized with B-actin. The mRNA and protein levels are relative to the control. (E) Assay for protein carbonyl content in tissue lysate using OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs). Data represent the mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

Trehalose intake improves autophagy in the retina of mice

In general, disaccharides are easily hydrolyzed into glucose molecules by vertebrates and have no known effect on autophagy in body tissues. Evidence, however, supports that orally administered trehalose in mice stimulates autophagy in tissues of the central nervous system, implying a bypass of the blood-brain barrier (Schaeffer et al., 2012). Despite similarities between the blood-retinal barrier (BRB) and blood-brain barrier (BBB) (Steuer et al., 2005), it is not yet known whether the oral intake of trehalose could induce autophagy in the retina. Two-month-old mice were administered 2% (w/v) trehalose in drinking water for 2 months to investigate the changes in the level of retinal autophagy. It was observed that the LC3-II level was upregulated in the trehalose-treated mice compared to those receiving only drinking water (**Fig. 6.4** A & B; unpaired t-test, $p < 0.05$), supporting the upregulation of autophagy in the retina by trehalose.

Trehalose intake and blood sugar level

The evidence of upregulated autophagy in the retina and CNS by trehalose, as well as its presence in the peripheral circulation of mice following oral intake, support that trehalose preserves its disaccharide form and not metabolized into glucose (Khalifeh et al., 2019). That notwithstanding, it is also true that vertebrates retain the active hydrolyzing enzyme trehalase in the kidney, liver and small intestines (Ishihara et al., 1997). Hence, it may be possible that prolonged trehalose intake could alter blood sugar concentration. To determine the effect of trehalose on the blood sugar level, 2-month-old mice were fed trehalose for 6 months and the fasting blood sugar and random blood sugar levels were measured. The random blood sugar increased by + 0.73 mmol/l ($p = 0.066$) and the 6-hour fasting blood sugar increased by + 0.64 mmol/l ($p = 0.074$) in the trehalose-treated group compared to control (**Fig. 6.4** C). These results showed that

prolonged trehalose consumption has a marginal effect on the blood sugar level which might be of less clinical significance.

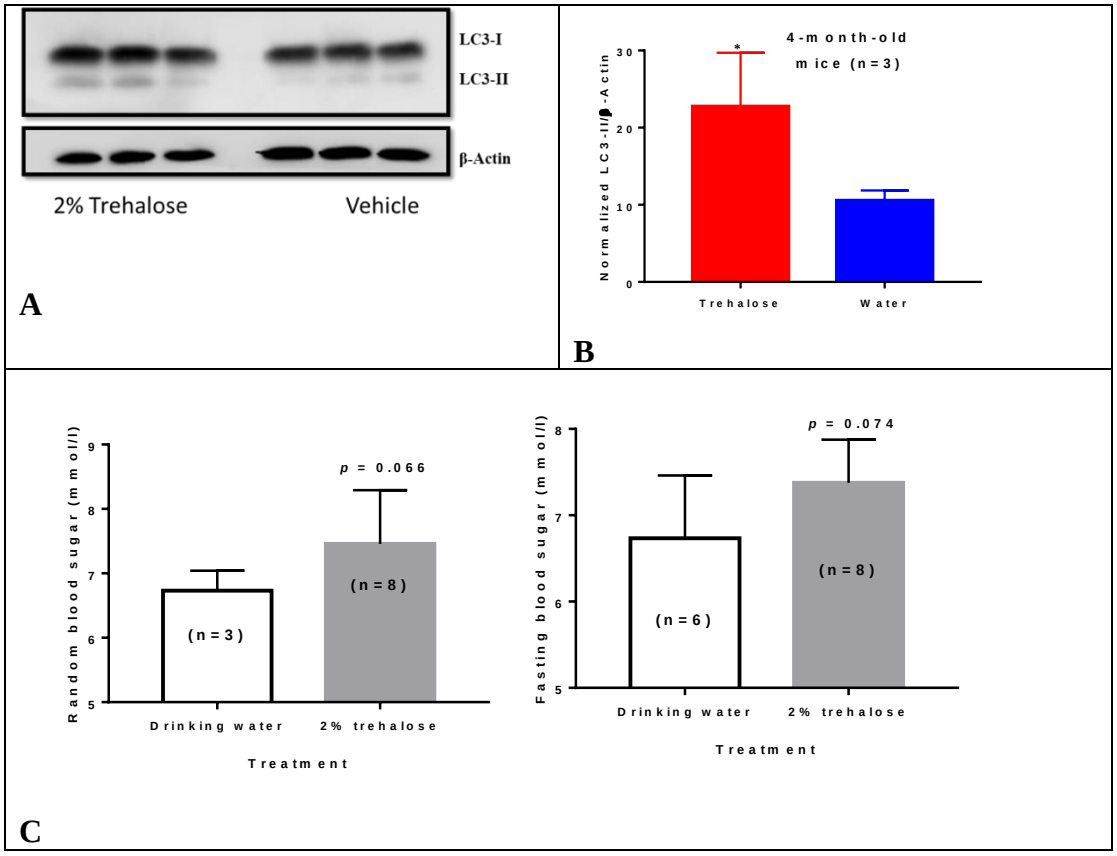


Figure 6. 4: Effect of trehalose intake on retinal autophagy and blood glucose level.

(A-B) Trehalose intake increased autophagy in the neural retina. Two-month-old mice were fed trehalose in drinking water ad libitum for 2 months or only water. (A-B) Immunoblotting to quantify changes in autophagy level in the neuroretinal tissue of mice. (C) Two-month-old mice were fed trehalose in drinking water ad libitum for 6 months or only water. Measurement of the random blood sugar (RBS) and fasting blood sugar (FBS) in blood collected from the distal tail vein of mice using the Accu Chek Performa Blood Glucose Meter. FBS was recorded at 2:30 p.m. after a 6-hour fast while the RBS was taken at 12 p.m. Data represent the mean ± SD. Statistical analysis was performed using the unpaired t-test. *$p < 0.05$ vs control.

Trehalose fed mice had improved ERG under photopic conditions

Based on the association between aging, impaired autophagy, and the retinal degenerative changes, it was hypothesized that upregulating autophagy could protect against retinal degeneration. To test the hypothesis, the baseline ERG of 2-months-old littermate mice was recorded prior to randomization for treatment with trehalose or vehicle. Repeated measurement of the ERG was performed when mice attained age 8 and 10 months. **Figure 6.5** compares the ERG recording in the older trehalose-treated mice and control (untreated mice) to the baseline data. Both the scotopic a-wave and scotopic b-wave amplitude was decreased with no difference between the treated mice and control at age 8 and 10 months (**Fig. 6.**5 A-D). Thus, autophagy does not affect the rod-mediated responses in the eye.

On the other hand, the mice receiving trehalose treatment recorded a higher photopic b-wave amplitude compared to the control at age 8 months (28%, two-way ANOVA, $p < 0.001$) and 10 months old (37%, two-way ANOVA, $p < 0.001$), although lower than the baseline (**Fig. 6.5** E & F). Since the photopic ERG protocol involved the presentation of stimulus over a rod-saturating background, achieved by a 7-minute light adaptation to 30 cd/m2 stimulus, the responses were reflective of the cone bipolar cell pathway (Kinoshita & Peachey, 2018). The OCT data, however, did not reveal any difference in the total retinal thickness and outer nuclear layer thickness between the trehalose-treated mice and control ($p > 0.05$). It was, therefore, unclear whether the improvement in the cone-mediated response was associated with any preservation of the cones, warranting further histological investigation in the control and treated mice to confirm these findings.

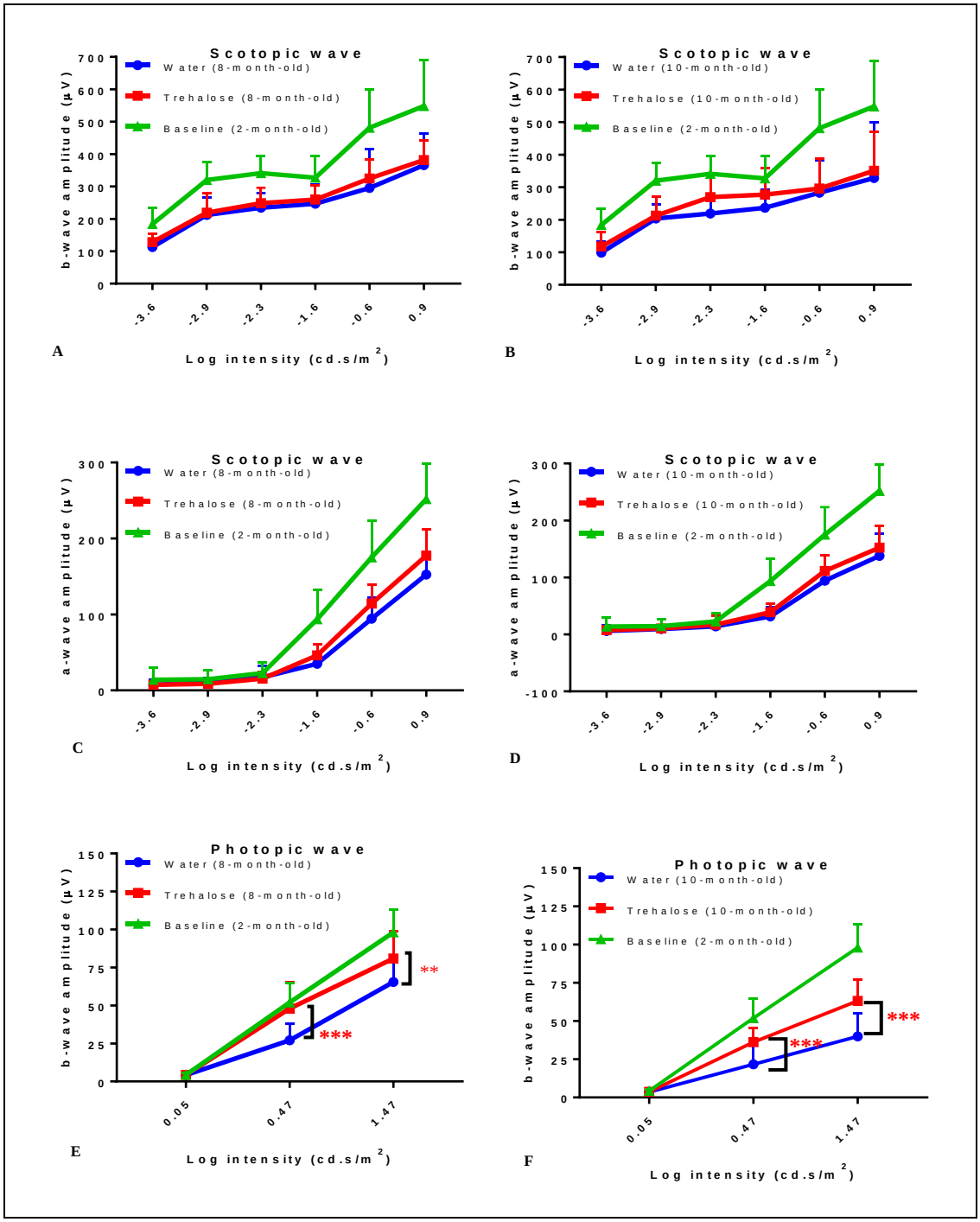


Figure 6. 5: Trehalose improves cone-mediated responses in aged BALB/C mice.

(A-F) Comparison of ERG measurement in mice on trehalose treatment vs. untreated mice at the age of 8 months (left panel) or 10 months (right panel). (A-D) Amplitude of the a-wave and b-wave recorded under dark-adapted conditions were similar between the trehalose-treated mice and untreated mice. (E & F) Amplitudes of the b-wave recorded under light-adapted conditions were higher in the trehalose treated mice compared to the untreated mice. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control (water).

Discussion

Aging is an important risk factor in the development of human retinal degenerative diseases with genetic or environmental underpinning, including retinitis pigmentosa (RP), diabetic retinopathy (DR) and AMD (Ardeljan & Chan, 2013; Tsujikawa et al., 2008). Aged human eyes show changes in retinal function and structure similar to AMD, albeit to a lesser extent, backing the proposition that AMD is an accelerated aging disease of the retina (Ardeljan & Chan, 2013; Ehrlich et al., 2008). Besides, the age-related retinal changes precede the development of AMD. Hence, an intervention delaying age-related degenerative changes or extending healthy lifespan might be useful in the treatment of AMD (Alavi, 2016; Franceschi et al., 2018; Luu & Palczewski, 2018). Investigation of the possible mechanism underlying the age-related retinal changes in the eyes of older mice, the dysregulation of TFEB-mediated autophagy and increased oxidative stress were found to be crucial. Moreover, it was demonstrated that orally administered trehalose improved autophagy in the retina and prevented the loss of cone photoreceptor function in aged wild-type BALB/c mice.

Retinal changes in normal aging

This study and many previous works support age-related changes in retinal morphology and function in the vertebrate eye (Aggarwal et al., 2007; Bell et al., 2015; Gresh et al., 2003; Nadal-Nicolás et al., 2018; M. A. Samuel et al., 2011). Most of the studies have claimed of generalized neuronal cell loss in the retina with age (Aggarwal et al., 2007; Bell et al., 2015; Gresh et al., 2003). But others have argued that the retinal thickness and functional changes are rather due to the shrinking of the cellular and synaptic layers as well as the linear increase in the retina with age (Nadal-Nicolás et al., 2018; M. A. Samuel et al., 2011). It is, therefore, likely that both neuronal loss and neuronal

reorganization underlie the functional loss and overall retinal thickness changes in the BALB/c mice.

Aging and oxidative stress

In our study, it was revealed that both macroautophagy and CMA were impaired, and oxidative stress increased in the retina of older mice with degenerative changes. Previous studies have implicated several molecular mechanisms, including mitochondrial malfunction, a decline in ATP production, intolerant glucose and lipid metabolism, increased oxidative stress and autophagy, for age-related degenerative changes in the retina or other nervous tissues (Bonomini et al., 2015; Payne & Chinnery, 2015; Ying Wang & Hekimi, 2015). The association between aging and increased oxidative stress in the retina has been well studied. The major contributors to increased oxidative stress in the aged retina include malfunction of mitochondria and the accumulation of toxic proteins and endogenous photosensitizers including lipofuscin and amyloid-β (Aβ) in the retina (Jarrett & Boulton, 2012; Payne & Chinnery, 2015). The accumulation of these substances interferes with retinal homeostasis and promoting age-related degenerative changes in the retina (Jarrett & Boulton, 2012; Jarrett et al., 2008). Thus, oxidative stress could be an essential component in the aging process (Jarrett & Boulton, 2012; Payne & Chinnery, 2015).

Role of TFEB-mediated autophagy inhibition in retinal degeneration

At both the cellular and organismic levels, autophagy induction by caloric restriction or pharmacologically was shown to slow down aging, extend lifespan promote and cell survival under harsh and stressful conditions (Madeo et al., 2015; Nakamura & Yoshimori, 2018), supporting the modulation of the aging process by autophagy. There

is also evidence to show that genetic inhibition of autophagy induces degenerative changes in mammalian tissues that resemble those associated with aging, and likewise, normal and pathological aging are linked to reduced autophagy activity (Rubinsztein et al., 2011). The regulatory role of autophagy in aging involves mitochondrial quality control and the regulation of protein homeostasis (referred to as proteostasis) (Ferrington et al., 2016; Moreno et al., 2018; Redmann et al., 2018). The removal of damaged mitochondria and proteins lowers the production of cellular reactive oxygen species (ROS) and prevents injury to the mitochondria itself (Moreno et al., 2018; Redmann et al., 2018). These clearly show that autophagy plays an essential role in aging and oxidative stress.

In addition, our study found the downregulation of the TFEB, a master autophagy regulator (Cortes & La Spada, 2019; Napolitano & Ballabio, 2016), in the retina of older mice. TFEB upregulates autophagy through the activation of the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network coordinating the different stages of autophagy (Sardiello et al., 2009; Settembre & Ballabio, 2011). TFEB overexpression promotes autophagosome formation, biogenesis of new lysosomes, and lysosomal enzymes for degradation (Sardiello et al., 2009; Settembre & Ballabio, 2011). In addition to autophagy, TFEB activation of the CLEAR gene network was reported to promote cellular catabolism of lipids, glucose homeostasis, and mitochondrial biogenesis (Sardiello et al., 2009; Settembre & Ballabio, 2011). These functions of TFEB are vital for the survival of neural cells, underscoring the impact of TFEB decline or upregulation on autophagy and the progression of neurodegenerative disease models, including Parkinson's disease, Huntington's disease and AMD (Cortes & La Spada, 2019; Martini-Stoica et al., 2016). In view of the vital

role of TFEB-mediated autophagy in the maintenance of retinal homeostasis, it is clear that its inhibition in the retina of aged mice underlies the increased level of oxidative stress and neurodegenerative changes.

Potential applicability of trehalose in neurodegenerative diseases

According to the data, orally administered trehalose induced autophagy in the neuroretina of mice of 4-month old mice that would likely have a normal BRB. This might indicate that the small disaccharide-molecule crossed the blood-retinal barrier (BRB) or has an indirect effect on the neuroretina from choroidal circulation. This outcome, while not previously reported, was not unexpected because there are evidence of autophagy upregulation in the brain tissue of mice fed trehalose (Schaeffer et al., 2012). Usually, the treatment of retinal diseases requires overcoming this barrier to deliver therapeutics to the retina and is usually circumvented through intravitreal injections (Campbell & Humphries, 2013). Therefore, determining whether trehalose can cross the BRB has the potential to revolutionize the treatment of retinal degenerative disease with evidence of impaired autophagy.

In addition, our study showed that prolonged trehalose intake did not have an adverse clinical impact on the blood sugar level, as there was < 1 mmol/l increase. This outcome is corroborated by the finding that trehalose ingestion did not induce rapid changes in blood glucose healthy humans (Yoshizane et al., 2017). It was previously reported that another mechanism by which trehalose induced autophagy in the presence of adequate nutrients or glucose involved blocking glucose uptake into liver cells, by inhibiting glucose transporters in the plasma membrane (DeBosch et al., 2016). Impaired glucose

uptake by the hepatocytes inhibits glucose metabolism and increases the blood sugar level (Adeva-Andany et al., 2016). Finding that the blood sugar level was not affected by a 6-month administration of Trehalose is reassuring of the less risk of causing any metabolic disorder. Currently, trehalose is regarded as a safe food ingredient for humans by the European regulatory system following approval by the U.S. Food and Drug Administration in 2000 (Richards et al., 2002). Trehalose could, therefore, be an autophagy inducer with broad application in the treatment of ocular diseases and neurodegenerative diseases in which impaired autophagy has been implicated.

Why does trehalose preserve cone function but not rods?

Our study showed that the trehalose-induced autophagy uniquely protected against loss of cone photoreceptors but not rods. Depending on the nature of retinal degeneration, either rods or cones are predominantly affected (Peter A. Campochiaro & Mir, 2018; Shelley et al., 2009; Veleri et al., 2015). Retinitis pigmentosa primarily affects rods at the onset of disease (Peter A. Campochiaro & Mir, 2018), whereas cones are more affected in AMD (Shelley et al., 2009). The unique patterns of photoreceptor loss in different forms of retinal degeneration may be an indication that cones and rods are vulnerable to differential cell death pathways, notwithstanding their interdependency on each other (arising due to physical contact or exchange of neurotrophic factors (Adler et al., 1999). Evidence shows that the cone photoreceptors are particularly vulnerable to oxidative stress (Peter A. Campochiaro & Mir, 2018; Narayan et al., 2016)**.** In fact, other reported promising antioxidant interventions for retinal degeneration, like trehalose, have been found to selectively preserve cones without much benefits on the rods (Aït-Ali et al., 2015; Komeima et al., 2006). This explains

why autophagy upregulation and reduction of oxidative stress by trehalose, therefore, contributed to the significant improvement in cones survival and function.

Conclusion

Altogether, our study elucidates that a decline in TFEB-mediated autophagy underlies the changes in retinal function and increased oxidative stress in normal aging. More importantly, it was demonstrated that trehalose intake induced autophagy in the retina and rescued the loss of cone function associated with normal aging.

CHAPTER VII

Conclusion

Summary of findings and conclusion

According to postmortem and *in vivo* experimental data, HQ-induced oxidative damage of RPE cells underlies age-related macular degeneration (AMD). Therefore, the first part of our experimental study sought to elucidate why the human RPE cells which are resistance to oxidative stress were prone to damage from the cigarette smoke oxidant hydroquinone. By *in vitro* investigations, we demonstrated that antioxidant and cellular homeostasis mechanisms including autophagy and the p62-NRF2 were dysregulated in RPE cells incubated with HQ. This piece of evidence corroborates the role of HQ exposure/cigarette smoking in the increased risk of RPE oxidative damage and AMD.

In dry AMD management, antioxidant vitamins and thiol supplements are recommended or prescribed as the mainstay treatment. It is, however, controversial whether antioxidant supplementation in AMD is protective or not. We premised our investigation on the argument that how a particular antioxidant affects the redox homeostatic mechanisms could determine whether it is beneficial in the prevention of RPE damage and AMD progression. Again through *in vitro* cell culture investigations, we found that autophagy and NRF2 were upregulated in human RPE cells cultured in growth medium supplemented with the antioxidant vitamins and thiols. Moreover, our results demonstrated that the induction of autophagy and NRF2 by antioxidants contributed to their protective role against RPE damage via oxidative stress. This molecular study provides a justification for the inclusion of antioxidant supplements,

particularly N-acetyl cysteine, and vitamin C, in the treatment of retinal degenerative diseases linked to increased oxidative stress.

Subsequently, we demonstrated that trehalose, a natural disaccharide molecule that induces autophagy via TFEB activation, could prevent oxidative damage in an *in vitro* RPE model of AMD. The relevance of TFEB activation and autophagy was then examined *in vivo* using a mouse of AMD. According to our data, aged mice with a decline in retinal TFEB expression and autophagy showed increased oxidative stress and abnormal morphological and functional retinal changes. These results, therefore, support TFEB-induced autophagy in the modulation of retinal homeostasis. Finally, we demonstrated that the oral administration of trehalose in mice led to the activation of TFEB and autophagy, resulting in the inhibition of oxidative stress and an improvement of the cone-mediated photoreceptor function.

In conclusion, these data demonstrate the antioxidative role of autophagy in the protection against oxidative stress-induced models of age-related macular degeneration.

Limitations

The earlier parts of the thesis focused on studies in cultured ARPE-19 cells. Hence, the lack of *in vivo* histological study and immunostaining examination of the RPE creates a gap that must be filled. This information would have been collected at the terminal stage of the longitudinal study after sacrificing mice. However, this study plan was disrupted due to the siege of The Hong Kong Polytechnic University by protesters. Laboratory animal studies have begun to address the shortcomings. And the investigations to be included are:

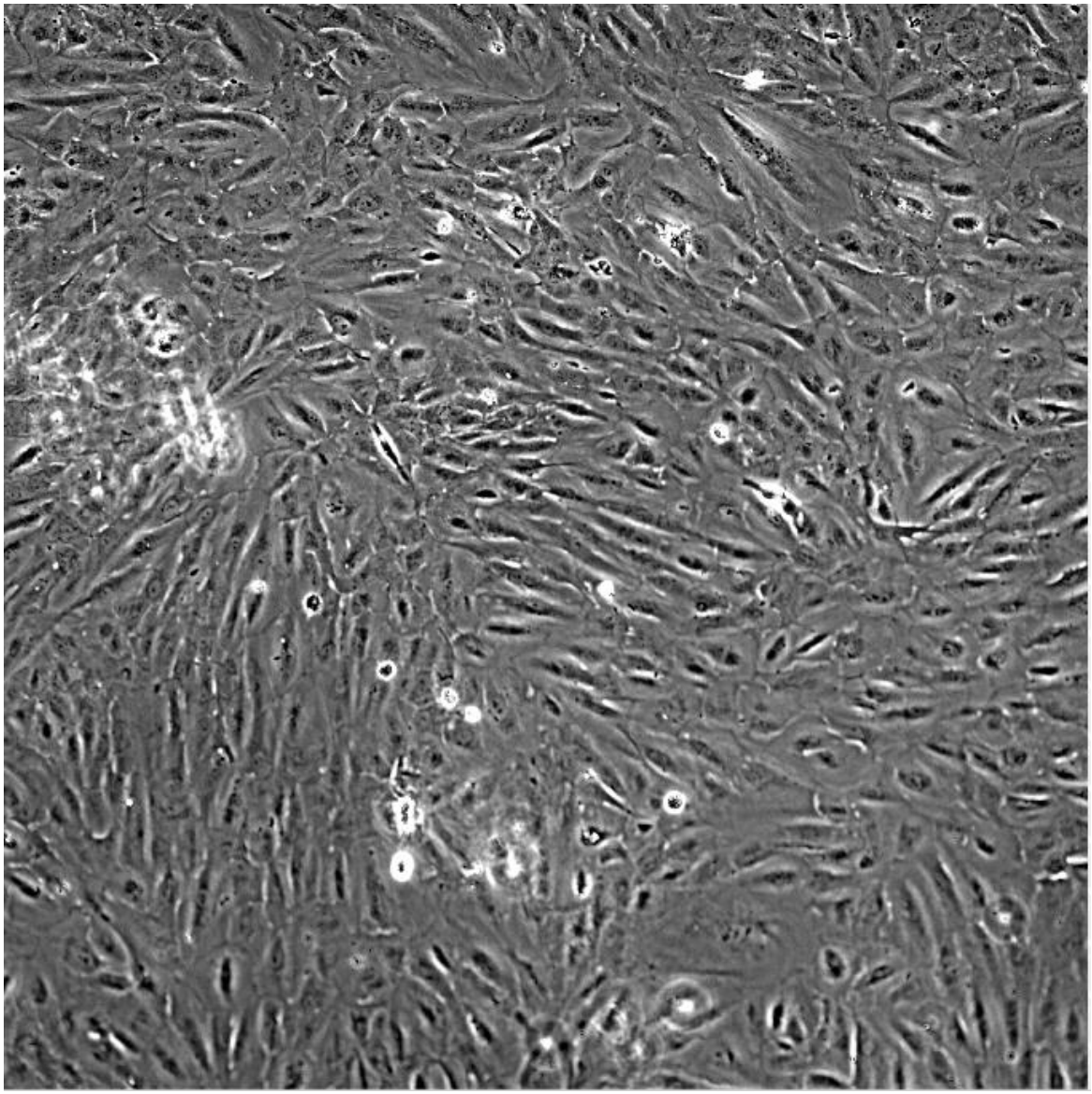
- examination of the retinal cytoarchitecture at the cellular level to determine changes in cell number in the ONL and cell death.
- immunohistochemical experiments to determine the location of the oxidative stress or autophagy in the retina or RPE.
- recording of the ERG c-wave to assess functional changes in the RPE.

Recommendations and future research

- Antioxidant vitamin supplements are already included in the management of age-related macular degeneration. Therefore, finding that these substances affect autophagy-lysosome pathway in an *in vitro* cell culture environment calls for the need to validate their effect *in vivo*.
- The safety and efficacy of orally administered trehalose in mice provide sufficient basis to recommend a clinical trial to test its therapeutic relevance in age-related macular degeneration.
- Also, there may be the need to investigate whether autophagy upregulation in specific retinal layers provide improved outcome compared to autophagy upregulation in the whole retina. This is because the retinal cells perform different functions that may not require similar levels of autophagy.

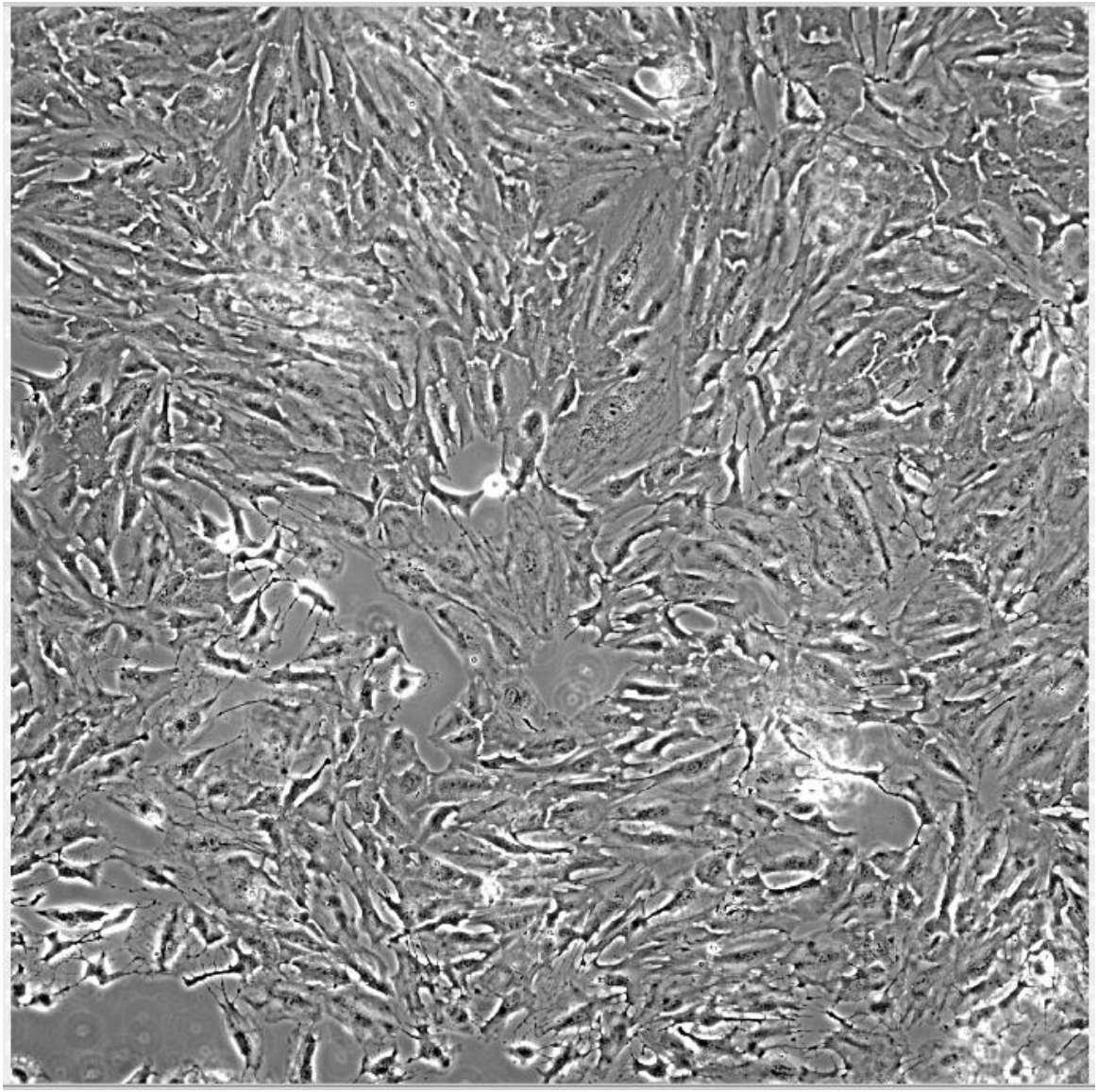
APPENDIX

Supplementary figures



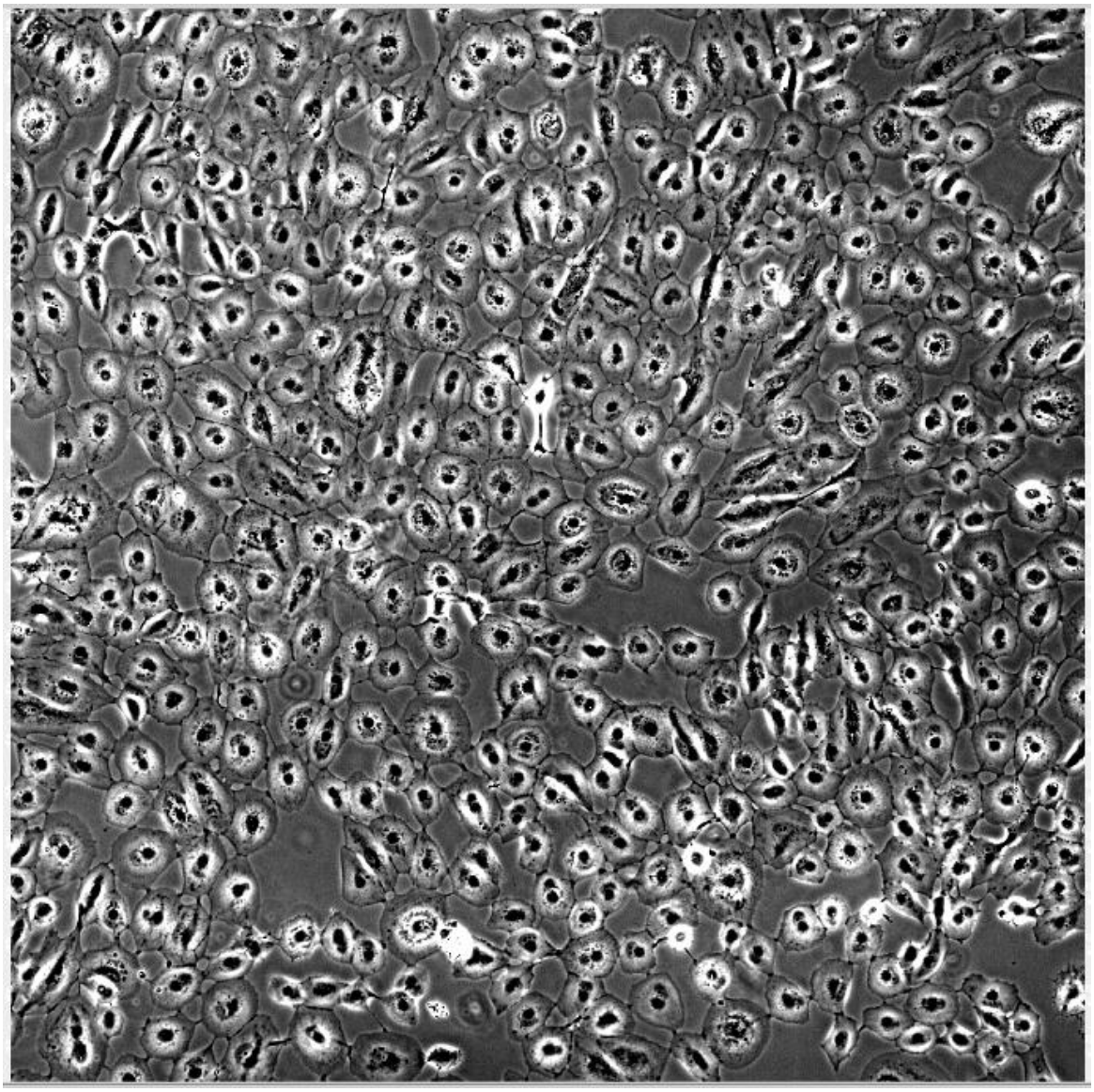
Supplementary figure 1**:** Healthy cultured human RPE cells in growth medium are spindle shaped and have a smooth outline.

ARPE-19 cells were grown in a confocal dish with serum-supplemented medium only without any other additional treatment. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



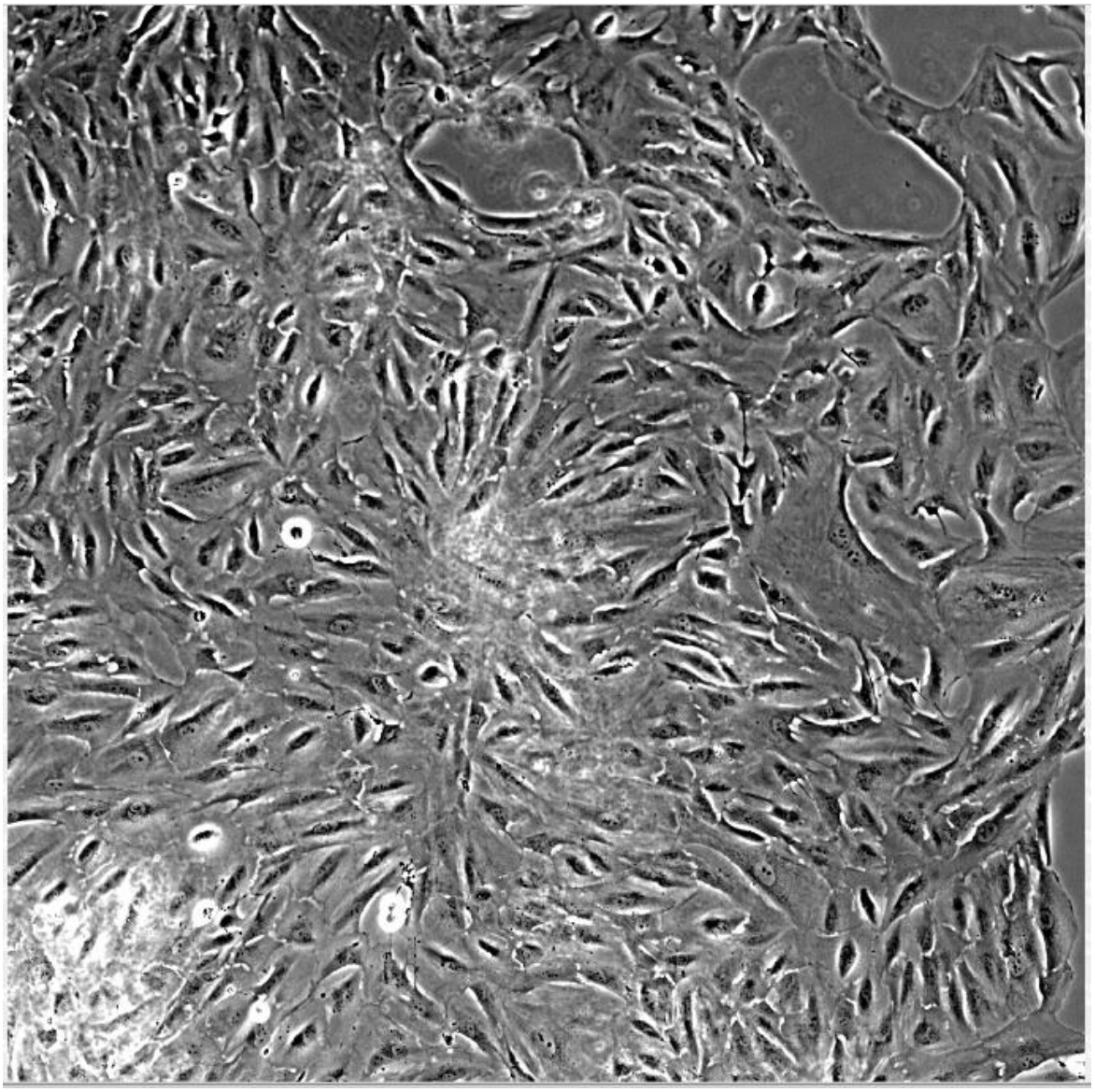
Supplementary figure 2**:** Human RPE cells become amoeboid-shaped and shrank in size when incubated with hydroquinone.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 20µM hydroquinone for 2 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



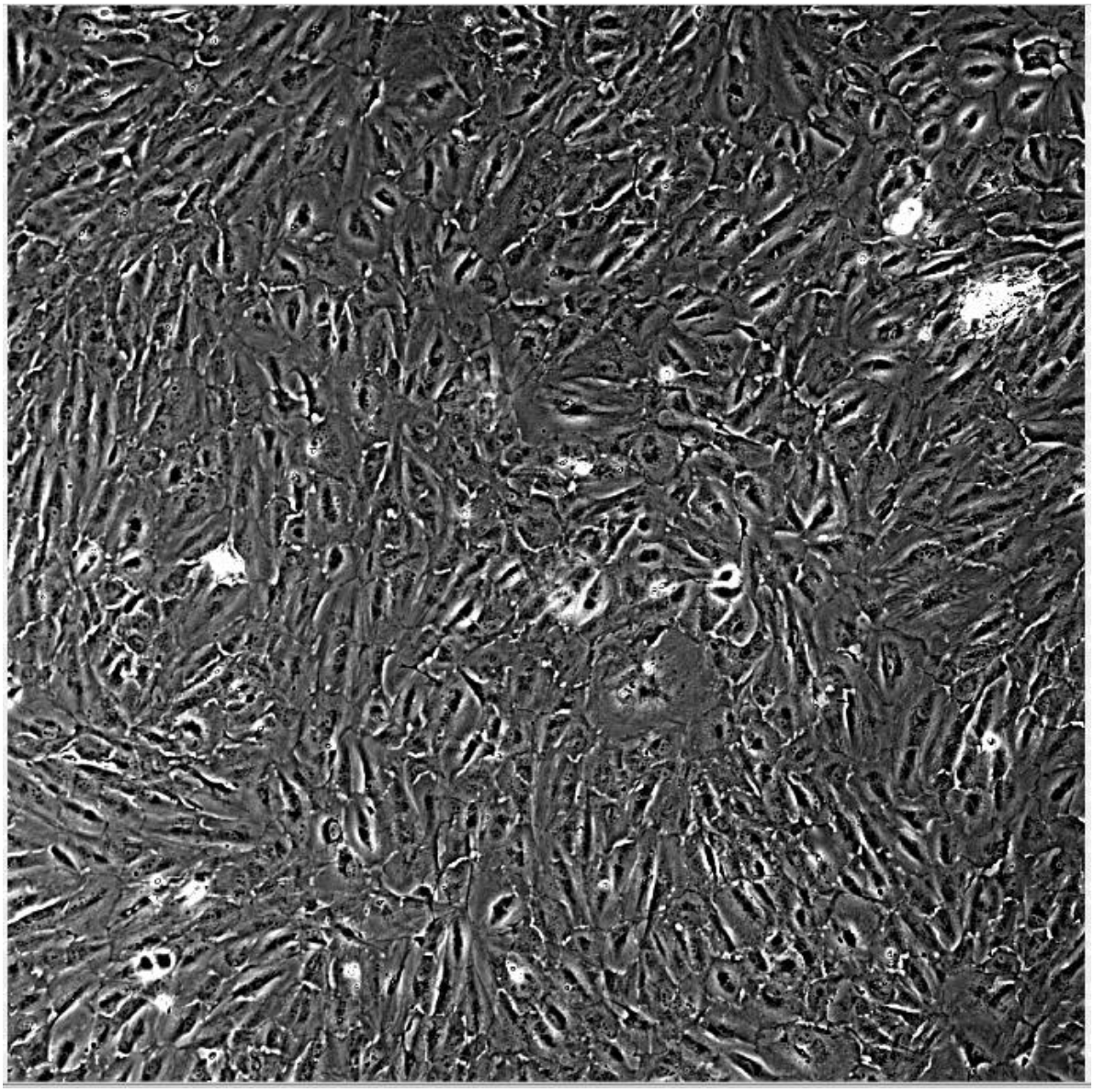
Supplementary figure 3**:** Human RPE cells become round shaped when incubated with hydrogen peroxide.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 500µM hydrogen peroxide for 2 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



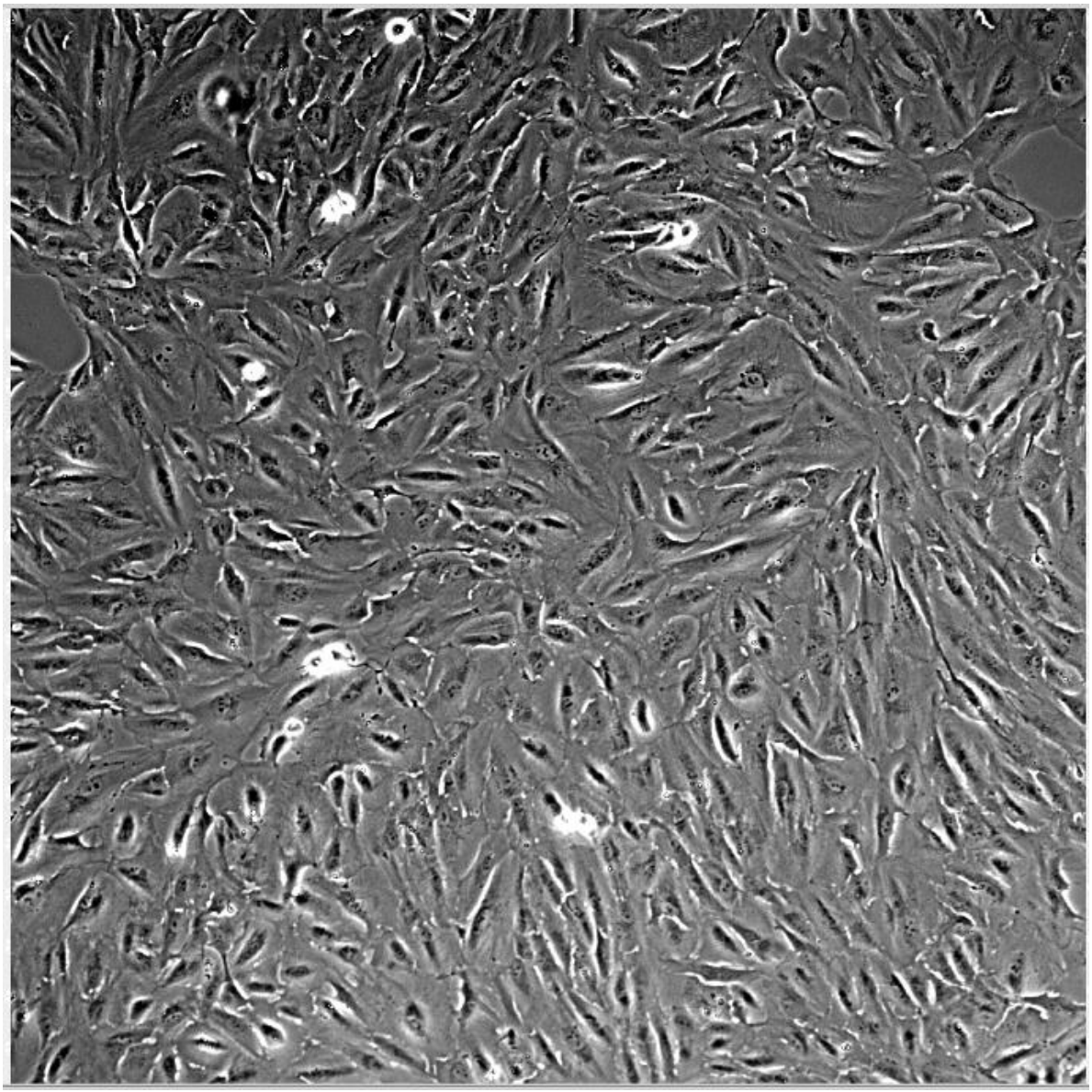
Supplementary figure 4**:** Human RPE cells maintain their normal spindle shape but become enlarged following glutathione (GSH) supplementation.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 500µM GSH for 24 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



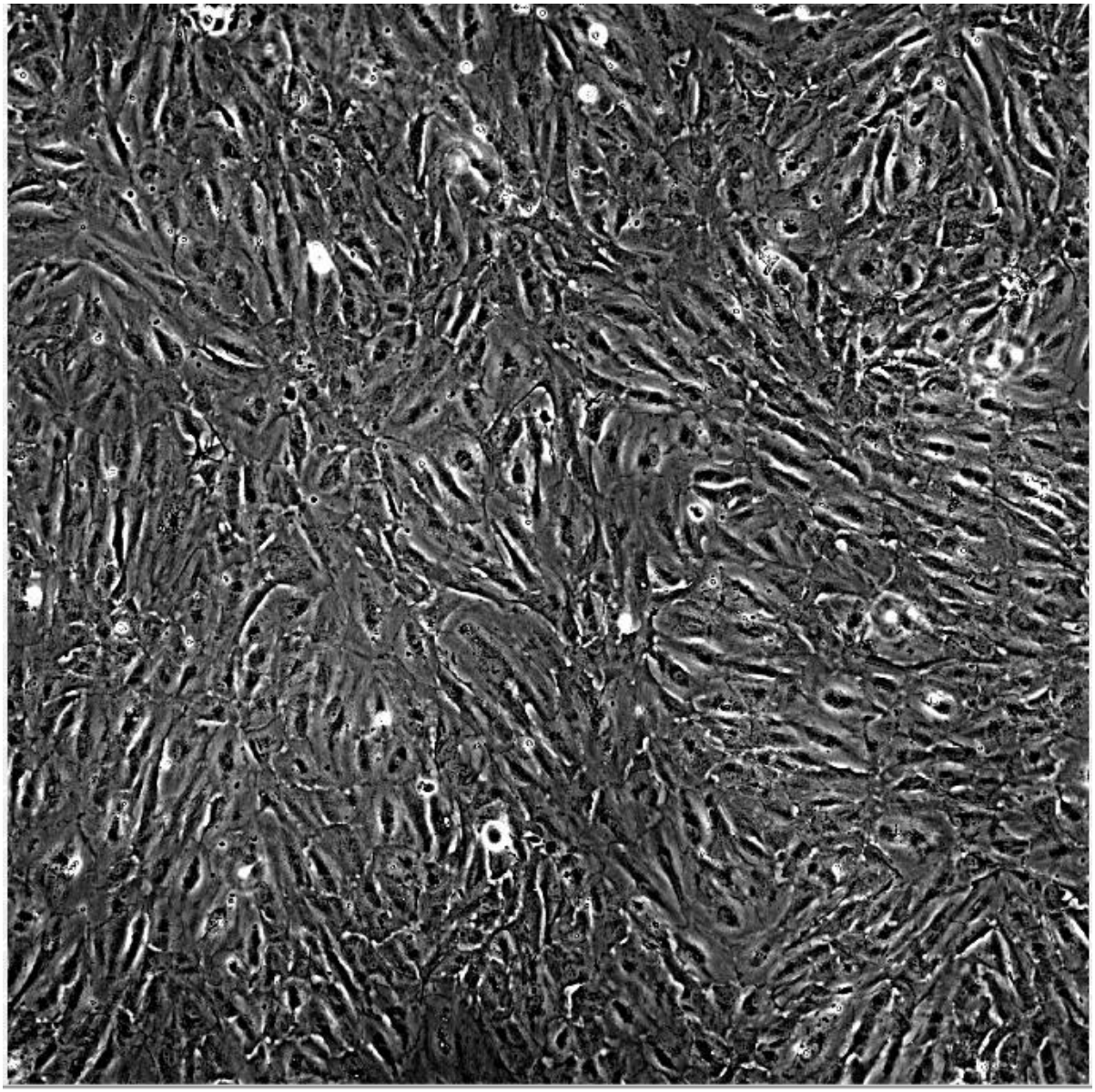
Supplementary figure 5**:** Human RPE cells maintain their normal spindle shape but show a mild reduction in size when supplemented with N-acetyl cysteine (NAC).

ARPE-19 cells were grown in a confocal dish in medium supplemented with 500 µM NAC for 24 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



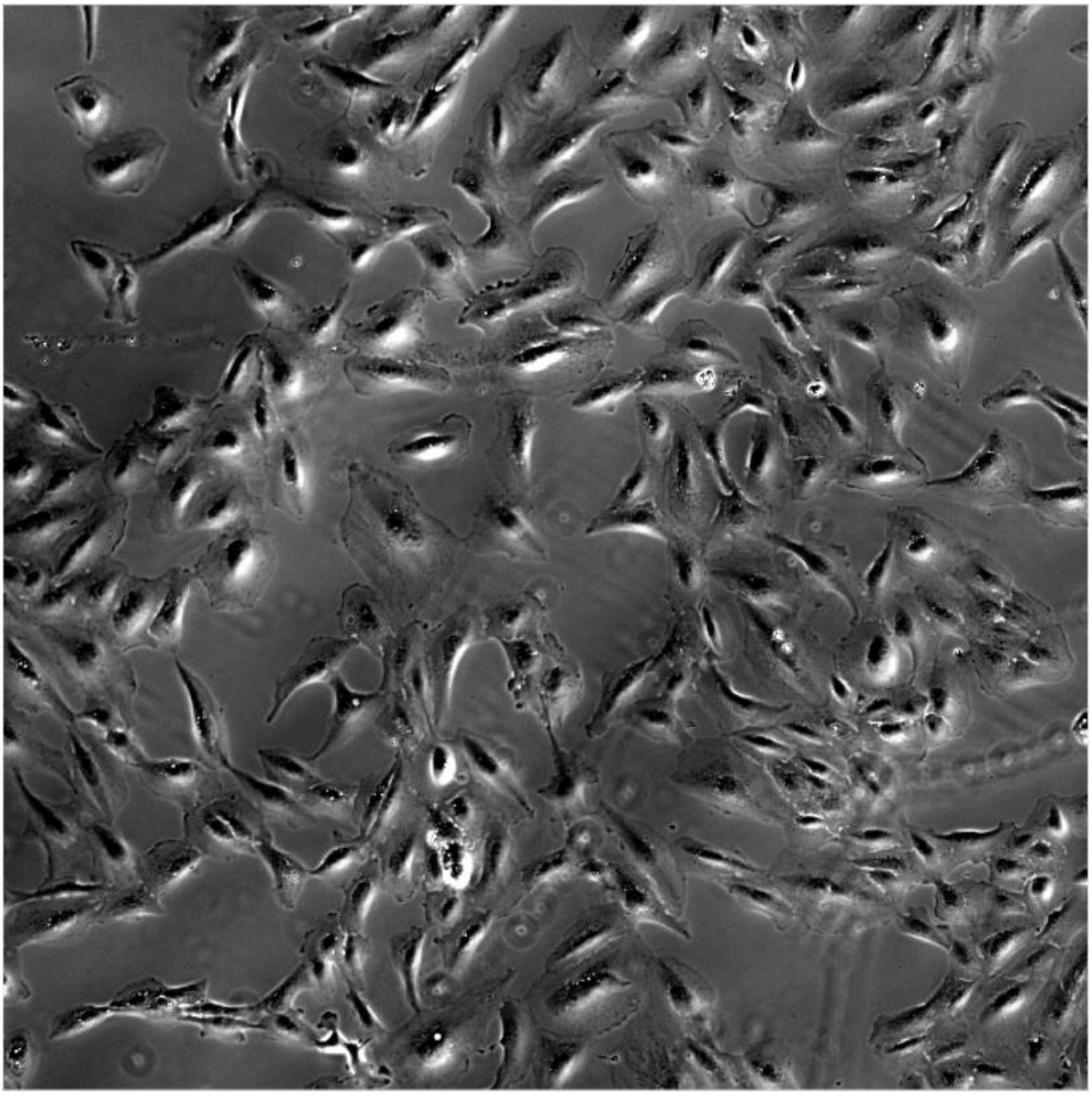
Supplementary figure 6: Human RPE cells supplemented with vitamin C show no changes in their normal spindle shape and size.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 500 µM vitamin C for 24 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification



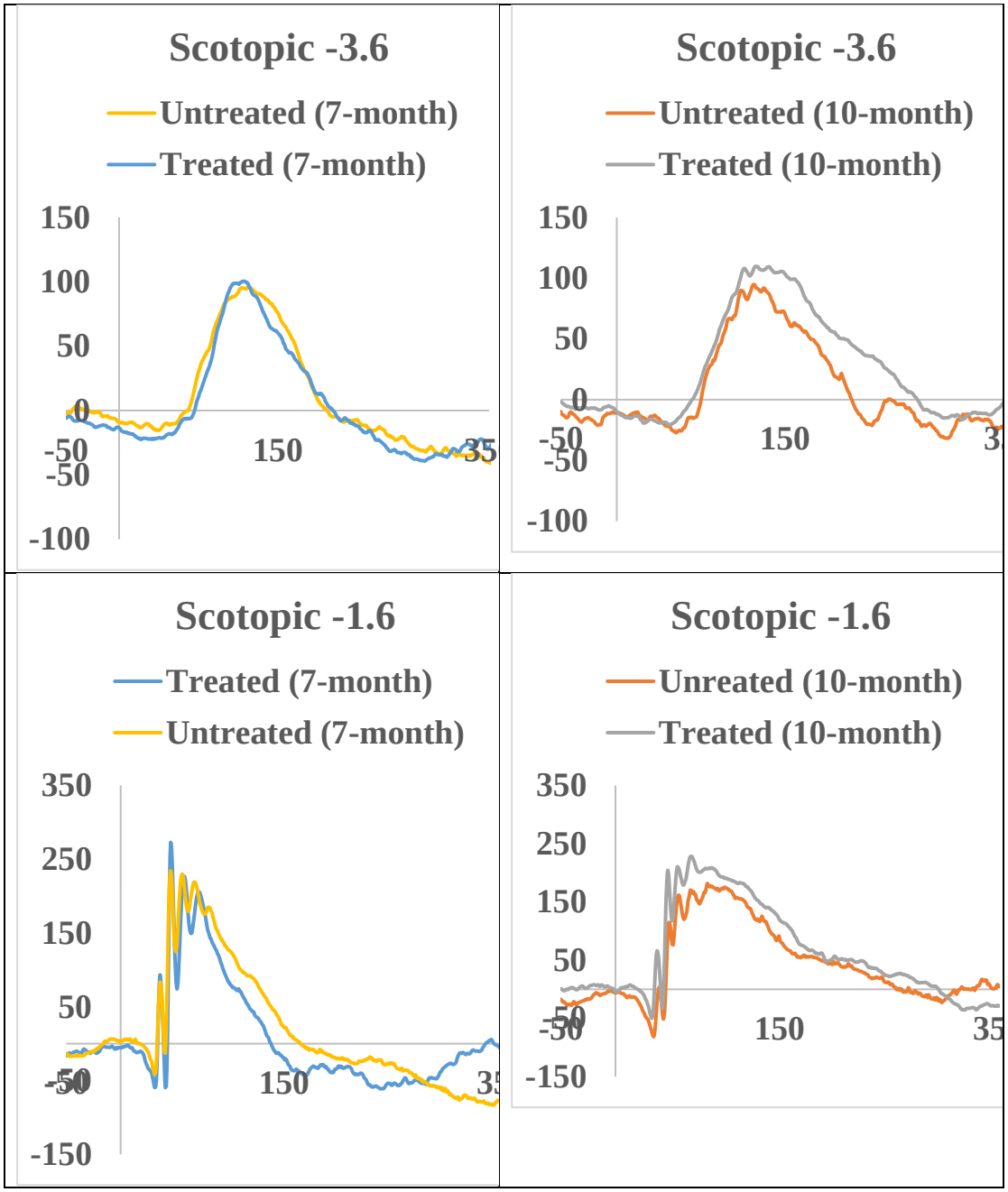
Supplementary figure 7**:** Human RPE cells supplemented with vitamin E show normal spindle shape and cell size.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 500 µM vitamin E for 24 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification

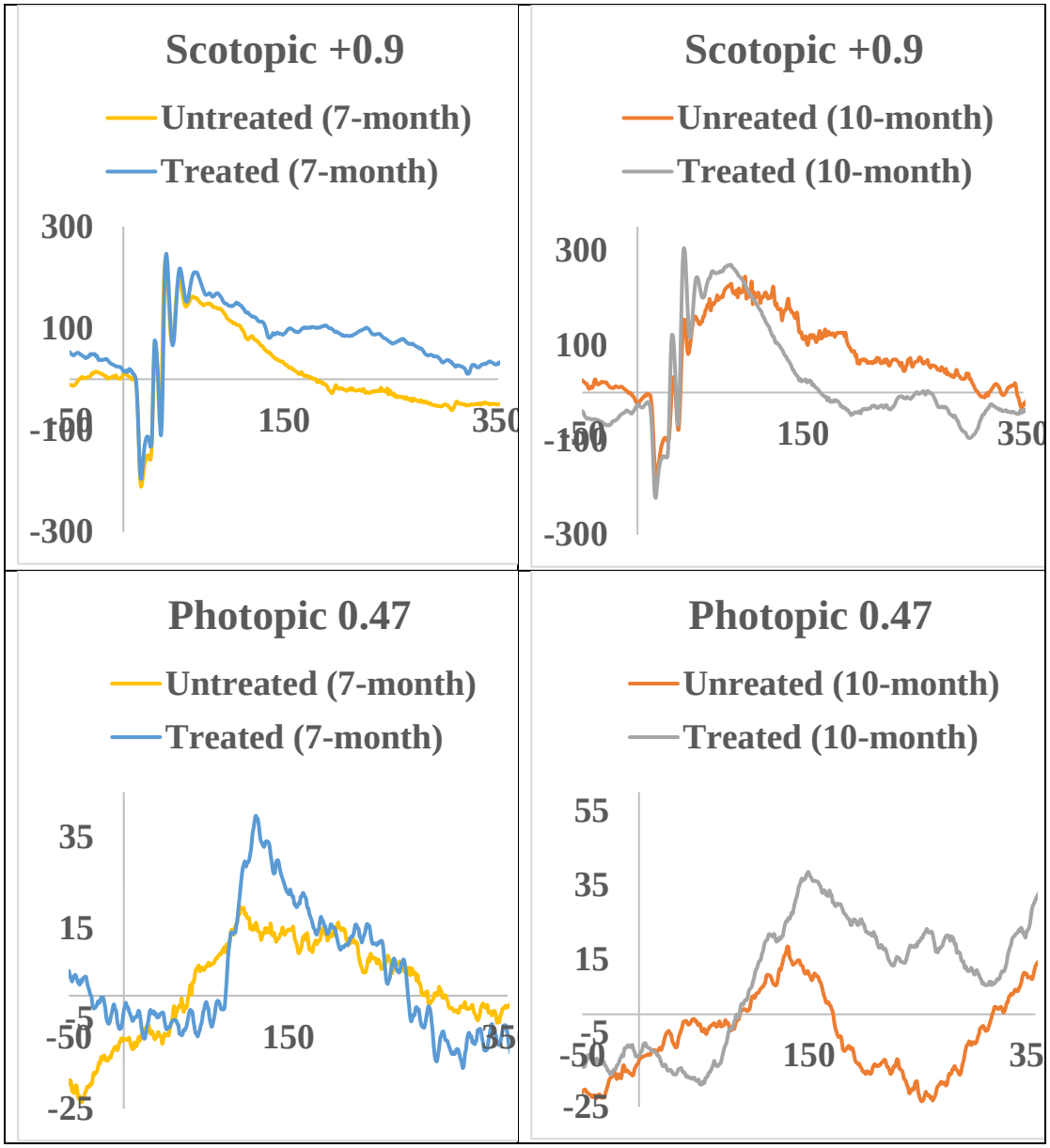


Supplementary figure 8**:** Human RPE cells maintain their normal spindle shape but become much enlarged following trehalose supplementation.

ARPE-19 cells were grown in a confocal dish with medium supplemented with 200 µM trehalose for 24 h. Live cell imaging was performed using an inverted confocal microscope with the phase contrast filter and 10X magnification.



Supplementary figure 9: Comparison of scotopic ERG responses for varied light intensities (cd.s/m^2) between BALB/C mice treated with trehalose vs. BALB/C mice treated with vehicle.



Supplementary figure 10**:** Comparison of the scotopic response at 0.97 cd.s/m2 and photopic ERG response at 0.47 cd.s/m^2 between BALB/C mice treated with trehalose vs. BALB/C mice treated with vehicle.

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