The Critical Role of AGEs/ALEs in Age-Related Dysfunction of the Retinal Pigment Epithelium

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Abstract

This study was to identify the critical role of advanced glycation and advanced lipoxidation end products in age-related RPE dysfunction in human eyes. Lipofuscin is an age pigment composed of incompletely degraded material derived from the photoreceptors and autophagy. With ageing, there is a gradual accumulation of lipofuscin pigment material in the lysosomal system within RPE that may have a direct influence on cell function.

Evidences appeared that AGEs/ALEs might play an important role in the formation of age-related intracellular fluorescence in RPE cells including that associated with lipofuscin granules. Also, they might appreciably alter the expression and enzymatic activity in the RPE lysosomal system, causing disruption of lysosomal function and having severe implications for RPE health in the ageing eye.

Our results showed there was a significant increase of auto-fluorescent granules accumulation in ARPE-19 cells after 28 days culture on glycated matrigel feeding with POS which means that AGEs/ALEs could increase lipofuscin accumulation within RPE in *in vitro*. These results were also confirmed by using primary RPE cells from 3 donors (male and female). Secondly, we also demonstrated that AGEs/ALEs accumulated within RPE could decrease Cathepsin D enzyme activities lead to cell dysfunction. Also Cathepsin B and APS activities were affected by AGEs/ALEs.

Results also showed that the lysosomal enzyme activities were deficient by AGEs/ALEs through altered the gene expression of the enzymes in the RPE respectively.

In summary, it has been demonstrated that the molecular and cellular function of the RPE could be transformed by non-enzymatic glycation.

Abbreviations

AGE	Advanced glycation end-products
ALE	Advanced lipoxidation end-product
AMD	Age-related macular degeneration
APS	Acid phosphatase
CAD	Cathepsin D
CAB	Cathepsin B
Cm	centimetre
DMSO	dimethyl suphoxide
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FCS	foetal calf serum
g	gram
g	gravitational pull of circular motion
HCl	hydrochloric acid
HEPES	N-[z-hydroxyethyl]piperazine-N'-[2-ethanosufonie acid]
Hour	hrs
Hz	hertz
Id	internal diameter
KCl	potassium chloride
KH_2PO_4	potassium phosphate
LF	lipofuscin
L	litre
M	molar
MeOH(CH_3OH)	methanol

$MgCl_2$	magnesium chloride
Mins	minutes
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimoles
N_2	Nitrogen
Na_2HPO4	sodium phosphate
nm	nanometre
OD	optical density
PBS	phosphate buffered saline (no Ca^{2+} or Mg^{2+})
PDGF	Platelet-derived growth factor
POS	photoreceptor outer segments
ROS	reactive oxygen species
RPE	retinal pigment epithelium
rpm	revolutions per minute
RT	room temperature
Secs	seconds
TCA	trichloroacetic acid
Triton X-100	t-octylphenoxypolyethoxyethanol
v/v	volume/volume
VEGF	Vascular endothelial growth factor
w/v	weight/volume
ul	microlitre
umol	micromoles

uM	micromolar
°C	Degrees centigrade

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1.1 General Introduction

Advanced Glycation End Products (AGEs) are a heterogeneous group of structures. They are formed over time by a nonenzymatic Maillard reaction between primary amino groups and carbohydrate-derived aldehyde groups on proteins (Niorge *et al.*, 1989). AGEs have been implicated in many of age-related diseases, such as: cataract, Alzheimer's disease and atherosclerosis. Handa and colleagues (2001) quantified there was an age-dependent increase of AGEs in human Bruch's membrane. They also identified AGEs were accumulated in basal deposits and drusen. From their report, they also determined that physiological dose of the AGE petosidine could up-regulate Platelet Derived Growth Factor β (PDGF-β) in retinal pigment epithelial (RPE) cells. This suggested that AGEs could influence the RPE phenotype.

Previous research showed that advanced glycation reactions appeared to play an important role in the age-related intracellular accumulation of autofluorescence and lipofuscin granules in post-mitotic epithelial cells (Stitt 2001; Vlassara *et al.*, 2002). Lipofuscin is a heterogeneous chemically modified material. It is lipid aggregates and recognized within the human RPE cell as yellow-brown refractive granules. It is characteristically auto-fluoresce under short-wavelength light excitation (Feeney 1978). Lipofuscin has been considered to be a biomarker of RPE cell ageing because it can accumulate within the lysosomal system in a variety of post mitotic cells throughout life (Boulton et al., 1990). Furthermore, lipofuscin has been demonstrated to be able to inhibit lysosomal enzyme activity *in vitro*.

It is not similar like the other cells in the body. Lipofuscin accumulation in

the RPE occurs through the autophagic breakdown of intracellular organelles. The major substrate for lipofuscin in the RPE of the eye is the un-degradable end product resulting from the phagocytosis of photoreceptor outer segments (POS) (Marmor *et al.*, 1998). To study the development of the RPE of *in vitro* models has primarily focused on the auto-fluorescent materials accumulated in the RPE cells after phagocytosis of POS, which are delivered to the cells through the culture medium (Boulton et al., 1990).

In vivo, the continual digestion of the POS requires a high level of lysosomal enzyme activity in the RPE. Cathepsin D is the dominant Cathepsin in the RPE cell and it plays the most important role in the digestive process. Previous research shows that it is about 80% of POS are digested in the RPE by the Cathepsin D (Constable *et al.*, 1997). Considering this large phagocytic load function, any effects causing the imbalance of Cathepsin D activity in the RPE cells could result in the accumulation of undigested POS phagosomes. This will ultimately cause morphological changes in the retina. To investigate the lipofuscin accumulation and other age-related changes in the RPE could help us to understand how ageing may adversely affect the function of the RPE cells.

1.2 The Retinal Pigment Epithelium (RPE)

1.2.1 Introduction of RPE

RPE is located between the neural retina and the choroids. It is a monolayer of cuboidal cells between the photoreceptors and Choriocapillaris of the eye and is specialized to uptake, phagocytize, and recycle the POS and retinaldehyde, the chromophore of rhodopsin (Osborne & Chader 1992). RPE cell death plays a major

role in the pathogenesis of age-related macular degeneration, the leading cause of blindness in the population >60 years of age in the developed world (Osborne & Chader 1992). Possible reasons given for the degeneration of RPE cells are their exposure to high oxidative stress and damaging irradiation.

The RPE in adults is a non-dividing system. It sustains a number of functions essentially for the preservation of photoreceptor cells (Zinn & Marmor 1979). The roles of RPE are: 1) establishment of a blood-retinal barrier, which controls the flow of metabolites and ions into and out of the retina; 2) phagocytosis and degradation of spent tips of photoreceptor outer segments; 3) absorption of optical radiation that has passed through the neural retina without being spent in the transduction process; 4) protection against free radical damage induced by high oxygen levels and light irradiation (Osborne & Chader 1992).

With ageing, the RPE in humans was seen to lose its light absorbing pigment-melanin and to accumulate the age-related pigment lipofuscin (Boulton *et al.,* 1990). These changes were accompanied by the progressive accumulation of deposits within the underlying Bruch's membrane (Boulton *et al.,* 1990). These age related changes in the RPE and Bruch's membrane have been implicated in the generation of Drusen and RPE detachment, which were associated with senile macular degeneration.

1.2.2 Structure and physiology of the RPE and Bruch's Membrane

The RPE is derived from the outer layer of the optic cup. It is continuous with the anterior pigment epithelium of the ciliary body (Mann 1969). RPE cells

are haxano-cuboidal and are relatively regular in shape throughout the retina. It also has some variation in size and shape across the retina (Hogan et at., 1971). The RPE cells are polarized epithelium. They face the photoreceptor side with the apical side but the basal side faces to Bruch's membrane (Hogan et at., 1971). Intracellular junction complexes are formed between neighbouring cells. They extend in a continuous fashion around the entire lateral membrane of each cell (Hogan et al., 1971). The junction complex consists of two portions: an inner component being the zonulae occludes (tight junctions) and the outer zonulae adherents. Lateral membranes of the RPE also exhibit large numbers of gap junctions. They facilitate the transportation of fluid and metabolites from cell to cell (Osborne & Chader 1992).

The RPE apical membrane is rich in two types of microvillus. One is a region that is highly complex in structure and rich in glycosaminoglycans (Bridges and Adler, 1985). It projects into the inter-photoreceptor space (Bridges and Adler, 1985). Long thin microvillus covers the majority of the apical surface (5-7μm length). It has no contact with the POS. However, the photoreceptor sheath, which is a more specialized form of microvillus, is directly associated with the POS.

The basal membrane of the RPE lies on a thin flat basement membrane (0.3μm thick) (Bridges and Adler, 1985). The topography of basal membrane does not follow the infolding of the basal invaginations. There are large numbers of organelles contained in the RPE cytoplasm such as smooth endoplasmic reticulum. They are distributed throughout the cell (Bridges and Adler, 1985). The nuclei are ovoid and located towards the base of the cell with large numbers of mitochondria.

Cigar-shaped melanin granules (size 1x 2-3μm) are prominent feature in the apical and mid-portions of the cell. Lipofuscin progressively accumulates in the

central to basal portion of the cell as membrane bound granules (1~2μm) (Osborne & Chader 1992). Golgi bodies, primary and secondary lysosomes and peroxisomes are also contained in the RPE cytoplasm.

The retina remains closely appose to the RPE. However, the mechanism of RPE-retinal adherences has not been established yet, although it was thought to consist of a number of elements that complement each other. These include the viscous nature of the inter-photoreceptor matrix. It offers resistance to separation of the two layers. Figure 1.1 (deGuileborn &Zauberman 1972).

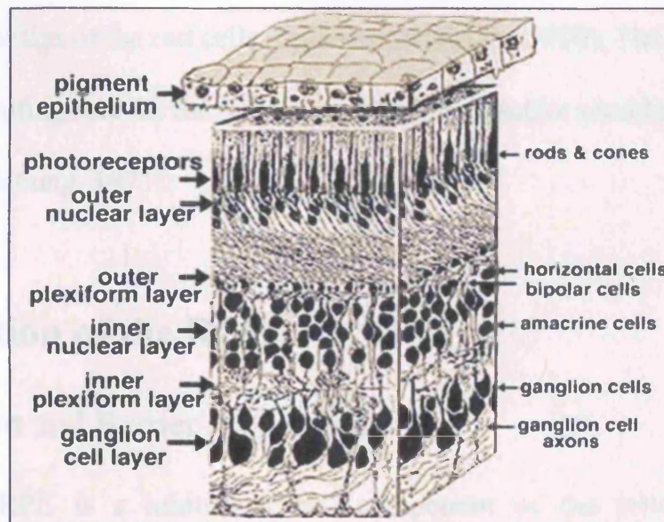


Figure 1.1 Cross-sectional views of RPE & neural retina. (deGuileborn &Zauberman 1972)

The RPE cell layer forms a selective barrier to the free diffusion of ions and metabolites through the continuous belt of tight junctions between the adjacent cells (Peyman et al., 1971). Thus, the molecular components of the extra-vascular fluid that leak from the fenestrated vessels of the chorio-capillaries are conveyed to the outer retina via a variety of selective trans-cellular transport systems.

It is part of a system of barriers which maintain the composition of the intraocular fluids. The trans-epithelial transport properties of the RPE have been investigated predominantly in amphibian and embryonic chick preparations. It has provided a wealth of information that the RPE has developed a number of specialized systems for the movement of ions, fluid, amino acids, retinoid and carbohydrates between the retina and the chorio-capillaries.

The RPE can remove the spent tips of POS by a poorly understood mechanism throughout life. This probably differs between rods and cones (Young, 1971). Some researchers suggested that there was an active ingression by the RPE sheath into the tips of the rod cells (Spitznas and Hogan 1970). The others proposed a simple engulfing role for the RPE in response to an active shedding of spent discs by the rods (Young, 1971).

1.2.3 The function of the RPE

1.2.3.1 Transport and Barrier Properties

The RPE is a multifunctional component of the retina. It performs numerous roles that are necessary for normal visual function. Many of the functional roles of the RPE are related to its unique location and to the metabolic, nutritive and barrier maintenance demands of the neural retina.

The RPE constitutes the outer blood-retinal barrier through its tight junctions. It forms a selective barrier to the free diffusion of ions and metabolites owing to the continuous belt of tight junctions between adjacent cells (Peyman *et al.*, 1971), and is responsible for the regulation and active transport of metabolites, such as glucose and amino acids from the choroidal circulation to the neural retina.

The barrier also has a function to prevent unguarded influx of blood cells and potentially harmful blood-borne proteins, for example, complements and immunoglobulins. Therefore, the RPE has responsibility for maintaining the microenvironment of the photoreceptors and the normal running of their surrounding matrix.

The trans-epithelial transport is achieved by various of specific membrane receptors on the apical or basal RPE plasma membranes (Hughes *et al* 1998; Bridges *et al* 2002,). For example, the RPE transports taurine to the sub-retinal space where it is specifically required by the photoreceptor cells (Miller & Steinberg 1976). Glucose is transported by GLUT1 glucose transporters, which are abundant in the apical and basal plasma membranes (Harik *et al* 1990). The RPE also regulates the passage of ions such as K^+ and Na^+ back and forth from the retina. This is important for the polarisation / hyperpolarisation of cell membranes and in the regulation of pH (Boulton & Dayhaw-Barker 2001).

It also helps to promote retinal adhesion by synthesis and preservation of the inter-photoreceptor matrix (IPM), which is composed of highly structured material that contains large molecules such as proteins and glycosaminoglycans. It is an important mediator of photoreceptor outer segment integrity. The matrix is firmly attached to the external limiting membrane and to the apical surface of the RPE. The adhesive properties of the IPM are determined by the hydration and ionic situation of the local environment. Because the RPE actively pump ions and water out of the sub-retinal space, it keeps the outer retinal ionic/osmotic in balance. Oxygen and other gases can freely penetrate through the choroid passing the RPE to the photoreceptors.

1.2.3.2 Antioxidant properties

The retina is particularly susceptible to cell damage by oxidative stress due to the presence of high oxygen tensions coupled with the continuous exposure to visible light which together provide ideal conditions for the formation of reactive oxygen species (Beatty et al 2000). Furthermore, photoreceptor outer segments contain high concentrations of polyunsaturated fatty acids that are readily oxidised (Bazan 1989, Miceli et al 1994, Tate et al 1995). The RPE has several defence mechanisms to protect against cellular damage by oxidative products with the first line of defence centred on limiting the intensity of light reaching the RPE. This is accomplished by macular pigment that filters out blue light (Beatty *et al* 1999) and by melanin that reduces overall light intensity (Sarna 1992).

The pigment melanin, found in RPE cells, is a heterogeneous polymer consisting of various monomers believed to be oxidation products of dopa (dihydroxyphenylalinine) derived from tyrosine (Sealy, R.C. *et al.*, 1980). Melanin contains intrinsic, indolesemiquinone-like radicals are reversibly photo-generated under visible or UV irradiation (Schmidt & Peisch 1986). RPE melanin serves a photo-protective role by absorbing radiation and scavenging free radicals and reactive oxygen species (ROS). Evidence also exists for a phototoxic role for melanin in RPE cells, especially in aged cells, including measurable ROS photoproduction. Apart from its photo-protective function, melanin in the RPE (and choroid) also plays an important role in helping to improve optical clarity by absorbing stray light to minimising light scatter within the eye.

The RPE is particularly rich in exogenous antioxidants, such as vitamin C, E, and endogenous cytoplasmic antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase and melanin , which can further quench reactive

oxygen species (Handleman & Dratz 1986, Newsome et al 1994). These provide the RPE with strong antioxidant protection.

1.2.3.3 The Phagocytosis and Degradation of Photoreceptor Outer Segment

Major function of the RPE is phagocytosis of rod and cone outer segment membranes (Elner *et al.*, 1981). It is a crucial process for the continual replenishment of new membranes and maintenance of the photoreceptor cell layer. Rods and cones continuously undergo the renewal of their outer segments. They are synthesised in the endoplasmic reticulum of the inner segment and then transferred to the base of the outer segment for assembly into new membranous discs (Young 1976). It is estimated that the turnover rate of rod outer segments is approximately 10 days (Young 1971). Over a 24 hour period, approximately 2000 – 4000 outer segment discs are ingested by each RPE cell (Young & Bok 1969).

Phagocytic uptake is achieved through the interaction of the apical processes of the RPE with the shed outer segment membranes. The precise molecular mechanisms involved in outer segment binding and phagocytosis by the RPE are complex and are not completely understood. But some evidences have suggested that a number of different ligand receptor systems are likely to be involved.

After phagocytosis, the phagosome contents are subjected to lysosomal degradation within the RPE. This process can be initiated by both light and endogenous circadian mechanisms. The intracellular digestion is a biphasic process. It initiates with small primary lysosomes fusing with phagosomes to form phagolysosomes. Subsequently, small lysosomes fuse with one another to form larger lysosomes, which then interact with the phagolysosomes probably through pore-like structures (Bosch *et al* 1993). Lysosomes contain a combination of over

forty degradative enzymes. Cathepsin D is considered to be the major protease responsible for degrading the rhodopsin rich disc membranes of shed outer segments (Regan *et al* 1980).

1.2.4 Ageing Changes in the RPE

1.2.4.1 Introduction

There are two symptoms of ageing in the eye, one is visual performance declined and the other one is progressively increasing incidence of disorders that affect most tissues in the eye (Marshall 1987). These ageing changes in the retina are thought to be major cause of blindness in the developed countries. For example: age-related macular degeneration (AMD). Many age-related changes in the RPE are readily identifiable by microscopy. Only recently there are more subtle changes identified, e.g. alterations in the spectral properties of pigment granules and lysosomal enzyme activities (Osborne & Chader 1992).

Most of the age-related changes in the RPE and Bruch's membrane are strongly associated with the development of AMD. However, the evidence between normal ageing changes and disease is not clear because many of the age-related changes seen in eyes with AMD can also be seen in advanced age and may precede the onset of AMD. Therefore, it is very likely that a complex interplay between genetic and environmental factors affect whether an individual is going to develop AMD or not (Boulton & Dayhaw-barker 2001).

1.2.4.2 Cell Morphology and Cell Death

Cell death is occurring in the RPE monolayer throughout life. It is an integral part of homeostasis and plays a major role in degenerative and proliferate

diseases (Hinsull and Bellamy 1981). There is a net reduction in the number of RPE cells across the retina with ageing even though the overall shape of RPE cells remain constant (Marshall 1987). Because the RPE is normally a non-dividing system, the insidious loss of macular RPE cells due to ageing will be replaced by adjacent cells spreading to fill in the gap. The increasing cell area is associated with an increase in the height of RPE cells (Feeney-Burn et al., 1984).

1.2.4.3 Melanosomes

The melanin content of RPE cells shows a regional distribution. It decreases from the equator to the posterior pole with a significant peak at the macula (Feeney-Burns et al., 1984). There is a significant decline in the total number of melanin granules observed in all regions after the age of 40 (Feeney-Burns *et al.*, 1984). When compared among three age groups (1-20, 21-60, 61-100), the decline in melanin content in the macular RPE between the early and late samples was about 25% (Osborne & Chader, 1992). Although the melanin granules morphology is similar in all age groups, there is an age-related change in their spectral characteristics (Boulton *et al.,* 1990).

1.2.4.4 Accumulation of Lipofuscin

The accumulation of lipofuscin is one of the earliest and most distinctive features of ageing in the RPE (Katz & Robinson 1984). Lipofuscin is an auto-fluorescent intracellular material. It is composed of an ill-defined mixture of lipid and protein. It can account for up to 19% of RPE cytoplasmic volume in the later stage of life (Feeney-Burns *et al* 1984). So far, chloroform soluble fluorophore A2E is the only successfully identified component (Eldred & Laskey 1993).

Lipofuscin can accumulate in many post-mitotic cell types such as neurones and cardiac muscle (Merry 1987), but is particularly apparent in ageing RPE. There is a common perception that the ageing RPE has a diminished capability to degrade phagosomal particles (Marshall 1987). This has lead to the suggestion that lipofuscin granules represent the undigested end products of outer segment phagocytosis and autophagy (Rakoczy *et al* 1992, Wassell *et al* 1998). There is a hypothesis supported by the fact that accumulation of lipofuscin granules is most concentrated at the posterior pole (Wassell *et al* 1998). This corresponds with the area of highest density of photoreceptor outer segments and so supports the suggestion that outer segments are the primary substrate for lipofuscin (Wassell *et al* 1998).

The formation of Lipofuscin is a complex process and two alternative theories have been proposed to account for it. Firstly, it has been suggested that POS are oxidised prior to phagocytosis. It results in the fact that it cannot be completely degraded by the RPE lysosomal enzymes (Kennedy *et al* 1995). However, some research discovered that although oxidative damage to outer segments seemed to be involved in lipofuscin formation, further modification within the RPE might also be required (Katz *et al* 1993, Wassell *et al* 1998). Secondly, it is possible that a decrease in lysosomal degradative capacity could lead to an accumulation of lipofuscin. This suggestion was supported by the fact that protease inhibitor treatment of RPE resulted in an accumulation of lipofuscin like material (Katz & Shanker 1989). It is possible that an age-related alteration in enzyme expression and/or activity could promote lipofuscin accumulation.

Evidence has shown that the accumulation of lipofuscin in macular RPE areas increases from 1% in the first decade of life to 19% in the 81-90 year age

group (Feeney-Burns *et al* 1984). Feeney-Burns' study also showed that the greatest increase in lipofuscin granules in the RPE actually occurred in the first two decades of life (Feeney-Burns *et al* 1984). Boulton (et al., 1986) reported that if lipofuscin accumulation reached a critically high level, the RPE cell death was increased (Boulton & Marshall 1986). This supports the comment that lipofuscin and other complex granules accumulation may compromise normal cellular activity by causing an overall reduction in functional cytoplasmic space. Therefore the available volume for organelles is reduced and the cell can no longer perform its functions (Marshall 1987). It has also been proposed that lipofuscin is photo-reactive (Boulton *et al* 1993, Rozanowska *et al* 1995). Winkler's (Winkler *et al* 1999). Research on a cellular system demonstrated that exposure of cells to both blue light and lipofuscin led to a decrease in cell viability, an increase in lipid peroxidation and a compromise of lysosomal integrity.

Lipofuscin accumulation in RPE mono cell layer has been thought to be associated with AMD. This pathogenic association is supported by the fact that both development of AMD and accumulation of lipofuscin are correlated with age. There are massive lipofuscin accumulations showed in the atrophic form of AMD (Sarks 1988). At present there is no direct evidence to suggest that lipofuscin is involved with AMD because although lipofuscin accumulation happens on all human eyes, not all eyes develop AMD. This suggests that other intrinsic and/or extrinsic factors may be also involved in the pathogenesis of this complex condition.

1.2.4.5 Lysosomal Enzymes

Previous research found that the lysosomal fraction from the RPE was three times more active in degrading rhodopsin than the lysosome fractions from other

cells (Zimmerman *et al* 1983). Moreover, it was also shown that in bovine RPE, the specific activity of acid lipase in the lysosomes was 10-fold higher than acid lipase activity in other bovine tissues (Hayasaka *et al* 1977) which represented an adaptation to the supra-high content of polyunsaturated fatty acids presented in the outer segments.

The lysosomal acid hydrolase's activity has a regional variation. This probably reflects regional inconsistency in environmental factors, such as phagocytic load of RPE locate in peripheral, central retina or macula. There has been evidence to show that the RPE underneath the macular region shows particularly high specific activities of acid phosphatase, Cathepsin D and arylsulfatase when compared to other areas of the retina (Hayasaka *et al* 1981). These findings are consistent with a more recent study that confirms higher acid phosphatase and Cathepsin D activities in human RPE derived from the macular region than cells derived from the peripheral retina (Boulton *et al* 1994).

This can be explained that the enzymatic activity increases in different regions because of the amount of material to be phagocytosed. Regional variation has also been observed in other non-human species. Such as bovine RPE cells from the area centralise which have a higher Cathepsin D activity than cells from the peripheral retina (Burke & Twining 1988). In canine cells, regional distribution of enzymatic activity varies (Cabral *et al* 1990), Cathepsin D is highest in the central retina and acid phosphatase, β-glucuronidase and n-acetyl-β-glucosaminidase are highest in the peripheral retina. It is obvious that, while there is some interspecies and regional variation, the lysosomal activity of RPE is highly specialised for lifetime degradation of POS. Previous research has shown that the lysosomal enzyme content and human RPE cell activity changed with age. The two lysosomal

enzymes (acid phosphatase and Cathepsin D) have an increase in overall activity with age, as studied in freshly isolated RPE cells.

1.2.4.6 Bruch's membrane

Bruch's membrane lies between the outer retina and its metabolic supply, and changes on the inner surface of Bruch's membrane are common finding in age-related retinal disease. Lipid accumulation in Bruch's membrane has been observed in the donor eyes with ageing (Pauleikhoff et al., 1990). The staining of lipid is most marked in the macular region of eyes from the over 60-old-year age group. The collagen fibres become more numerous with age and the fibres' atypical banding periodicity changes are also apparent in the Bruch's membrane fibrous layer (Grindle and Marshall, 1978).

1.2.4.7 Drusen

As previously outlined, there is a potential link between accumulation of lipofuscin and the development of AMD. However the gradual accumulation of lipofuscin granules within the RPE is accompanied by build up of abnormal extracellular deposits beneath the RPE within Bruch's membrane. This has lead to an alternative and increasingly strengthening theory that age-related changes in Bruch's membrane could also be associated with development of AMD and other outer retinal degenerative disorders.

Most of the changes to Bruch's membrane involve accumulation of abnormal material in the form of either discrete or diffuse deposits. These deposits, known clinically as drusen, constitute accumulations of extracellular material lying between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane (Sarks *et al* 1994). Numbers of drusen

have been correlated with increasing age (Sarks *et al* 1994). Where their accumulation may serve to elevate the RPE cell layer from Bruch's membrane causing localised areas of detachment (Schwatrz *et al* 1998). Although the precise composition of drusen remains to be elucidated they are known to be composed mainly of mucopolysaccharides and lipid components, and Hageman's report had shown that widespread drusen were the earliest ophthalmoscopic indicator of AMD initiation or progression and could be classified as hard or soft, discrete or confluent (Hageman & Mullins 1999). Hard drusen originate from the discharge of membranous debris from the RPE cells and reflect a localized disorder of the RPE (Green *et al* 1985). On the other hand, soft drusen are an indication of diffuse RPE dysfunction with pathophysiological impact on AMD initiation and progression (Green *et al* 1985). Figure 1.2

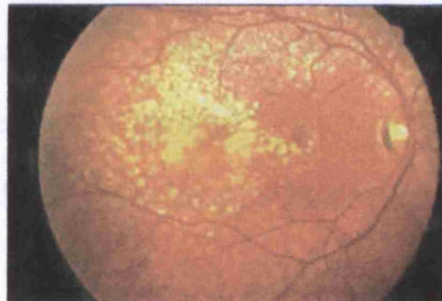


Figure 1.2 Fundus Photograph showing drusen accumulation (Green et al., 1985)

Drusen, the clinical discrete spots, are a common finding in the macular region and in the far periphery of elderly eyes (Eagle, 1984). Hard drusen occurs in 80% of post-mortem eyes (Coffery and Brownstain, 1986) and soft drusen with less well defined boundaries is usually larger than hard drusen and rarely occurs before the age of 55 (Garner et al., 1994). Histochemical studies show that the fluorescein-excluding drusen are composed of lipids and protein deposits (Marshall

1987). To date, there is no significant difference in the molecular composition of hard and soft drusen that have been identified which suggests that they may have a common origin (L.V. Johnson et al., 2003).

In advanced stages of AMD, hard drusen can coalesce to form confluent plaques that are associated with the atrophy of large patches of RPE and macular regions of retina (geographic atrophy) (Sarks SH 1982). Large numbers of drusen or large confluent drusen are also associated with a significantly increased risk of developing choroidal neovascularization, characteristic of the exudative (wet) form of AMD (Green WR et al., 1994).

1.2.4.8 Antioxidant Status

There has been much evidence to show that antioxidants implicated in the ageing process, and the antioxidant status, of the RPE is altered with age. Friedrichson's research showed that the levels of the non-enzymatic antioxidant Vitamin E in macular RPE declined with ageing (Friedrichson *et al* 1995); Liles research showed that there was an age-related increase in activity of the antioxidant enzyme catalase in the RPE, but there was no change found for the RPE's superoxide dismutase activity (Liles *et al* 1991). Antioxidant enzyme activity has also been correlated with lipid peroxidation as a function of age (Castorina *et al* 1992). A progressive insidious change in antioxidant status could be a potential mechanism for age-related RPE cell dysfunction as it has been shown that oxidative stress can cause dysfunction and death in cultured RPE (Cai *et al* 2000).

1.2.4.9 Advanced Glycation

Advanced glycation endproducts (AGEs) have been implicated in the pathogenesis of a range of disease processes; the best known and most researched

process is the role of AGEs in the development of the vascular complications of diabetes (Vlassara *et al* 1994; Stitt *et al* 1999, Jenkins *et al* 2002). However, more recently AGEs have also been linked with other diseases and degenerative disorders associated with ageing including atherosclerosis (Stitt *et al* 1997a, 1997b, 1997c), Alzheimer's disease (Munch *et al* 1998, 2002), male erectile dysfunction (Jiaan *et al* 1995, Seftel *et al* 1997), cataract formation (Harding & Crabbe 1984, Stevens 1978), osteoarthritis (DeGroot *et al* 2001) and pulmonary fibrosis (Matsuse *et al* 1998). Since the RPE and Bruch's membrane are known to undergo significant changes with ageing and the fact that age is the only certain risk factor for development of AMD, it is possible that advanced glycation may play either a contributory or facilitative role in development of age-related RPE dysfunction.

1.3 Age-related Macular Degeneration (AMD)

1.3.1.1 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the Western world. It is the typical ageing changes that occurs in the RPE and Bruch's membrane. It was responsible for approximately 95% of blindness and partial sight registrations in the UK (O'Shea 1998). Klein said this disease was prevalent in the elderly population (Klein *et al* 1992) and would become more common due to the fact that people would be living longer.

The Blue Mountains Eye Study in 1995 (Mitchell *et al* 1995) found that AMD percentage rose from 0% among people younger than 55 years of age to 18.5% among those over 85 years of age. It was estimated that the percentage of

people affected by AMD would almost double in the next 25 years (Stone *et al* 2001). With an increase in elderly population in the future, there is no doubt that AMD will become a substantial social and public health problem in most Western societies (Klein *et al* 1992). The cause of AMD is still not clear, and the disease is extremely difficult for the ophthalmologist to treat in the clinic.

1.3.1.2 Concept of AMD

There has not been a universally accepted definition for AMD. Previously, doctors and scientist tended to use the term of senile macular degeneration to describe many a variety of changes in the macular region. Subsequently, the term age-related macular degeneration was adopted and referred to a group of late stage clinical symptoms that affect the macula.

The difficulty to define a concept for AMD is because AMD has a wide range of clinical features consistent with the diagnosis of AMD. The International Age-related Maculopathy study group proposed a classification system for early and late stages of the disease in 1995 (Bird *et al* 1995). They suggested that the term of age related maculopathy would be used to describe the early stages of the disorder which were characterised by drusen formation and pigmentary abnormalities, whereas they recommend that the term AMD would be reserved for later stages of the disease. AMD can then be further classified as the dry form (non-exudative) and the neovascular form (exudative) (Bird *et al* 1995).

1.3.1.3 The Macular Structure and Function

The macula is a circular zone located within the central retina with a radius of approximately 3mm, at the centre of which is the fovea (Young 1987). It is defined by a region of yellow pigmentation, and is also called yellow spot. The

anatomical structure of macular has a number of differences compared with the rest of the retina. For example, the macula consists largely of cone photoreceptors. The photoreceptors density is highest in the retina. It also has a large number of populations of ganglion cells (Penfold *et al* 2001). The feature of having both the highest densities of photoreceptors and ganglion cell enables the macular to have high-resolution vision and acuity (Penfold *et al* 2001). As a result the macula is the area where the RPE is most metabolically active (O'Shea 1997).

The fovea region is located at the centre of the macula. It is the thinnest part of the retina as the inner nuclear layer. Ganglion cell layer and retinal blood vessels are displaced to ensure that there is no interference to the light that reaches the photoreceptors. In patients with AMD, it was estimated that about 90% of subretinal neovascular membranes are found close to the centre of the fovea (Berkow *et al* 1984). Therefore the highest visual acuity is affected by AMD.

1.3.1.4 The features of AMD

Histopathology and clinical pathology studies have evaluated the various morphologic features of AMD (Green *et al* 1985, Green & Enger 1993, Green 1999). Drusen formation and extracellular deposits beneath the RPE are the characteristic markers of AMD which cause pigmentary abnormalities and hyperplasia of the RPE. Significantly, all the major features of dry AMD can also be seen in people with normal ageing and are present in some form in almost all aged eyes, albeit to a lesser extent. The processes by which these changes occur are not well understood; the pathogenesis of AMD is complex and remains to be elucidated. It is highly probable that accelerated age-related changes in the RPE and Bruch's membrane play a key role in the development of this disorder.

1.3.1.4.1 Dry AMD (non-exudative)

AMD is either non-exudative (dry AMD) or exudative. Non-exudative or dry maculopathy is the most common form of AMD, accounting for 80-90% of cases (Sharon et al., 2002). Dry AMD is caused by the progressive degeneration of the RPE associated with secondary loss of the photoreceptor cells and results in either moderate or severe loss of central vision (Sunness 1999) (figure 1.3). Loss of the photoreceptors and RPE occurs slowly over time and consequently vision loss occurs as a gradual process.

Geographic atrophy of the RPE is the advanced form of non-neovascular AMD (Sharon et al., 2002). It is responsible for approximately 20% of the legal blindness caused by AMD, compared to choroidal neovascularisation accounting for the remaining 80% (Ferris *et al* 1984). At the present time there is no effective form of treatment.

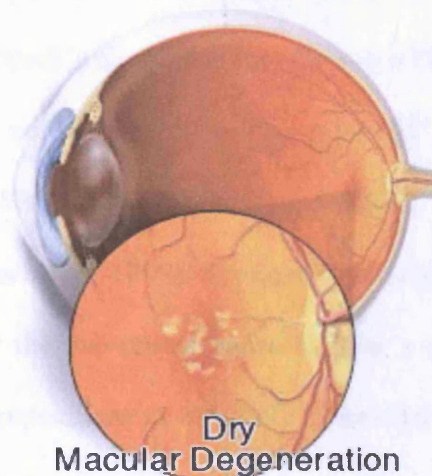


Figure 1.3 The yellowish deposits in the macula, known as drusen, are a classic sign of dry macular degeneration. The dry type is much more common and is characterized by drusen and loss of pigment in the retina. (Sharon et al., 2002)

Clinically it consists of one or more areas of RPE hypo-pigmentation with exposure of the underlying choroidal vessels (O'Shea 1998). Histological studies demonstrate that the RPE and photoreceptors are lost in the area of geographic

atrophy (Sunness 1999). It is presumed that RPE degeneration occurs first, followed by secondary choriocapillary atrophy (Korte *et al* 1984); however, it has also been hypothesized that choroidal vascular dysfunction could be a primary event that leads to subsequent degeneration of the RPE. This theory is supported by recent evidence, which has shown that choroidal blood flow is compromised in dry AMD (Grunwald *et al* 1998).

1.3.1.4.2 Wet AMD (Neovascular AMD)

Exudative AMD is also known as "wet" or neovascular macular degeneration. In contrast to dry AMD, vision loss occurs much faster in patients with the neovascular form of the disease and central vision lost may be significant over a period of days (Bird *et al* 1995). Although they may also extend into the subretinal space, Neovascular AMD is caused by the rapid invasion of choroidal blood vessels through Bruch's membrane into the sub-RPE space (Green & Enger 1993). Such choroidal neovascularisation causes sub-RPE haemorrhage resulting in an accumulation of blood or serum beneath the RPE and may lead to a RPE detachment (Campochiaro *et al* 1999). Serum or blood may then pass through the detached RPE to enter the sub-retinal space causing a serous or haemorrhagic retinal detachment (Campochiaro *et al* 1999). Choroidal neovascularisation can then produce a fibrovascular disciform scar.

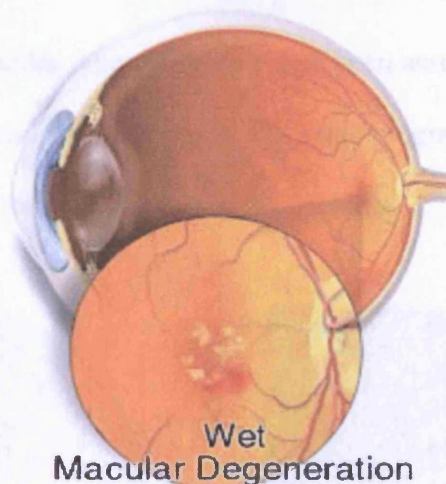


Figure 1.4 A tiny net of abnormal blood vessels has developed under the retina, causing bleeding and loss of central vision (Green & Enger 1993)

This occurs when there is a proliferation of RPE cells associated with the new vessels. Sub-RPE or sub-retinal haemorrhage stimulates the scarring process by promoting the proliferation of RPE cells and fibrous tissue beneath the new retina (Green *et al* 1985). The scar replaces the outer retina and then causes a permanent central scotoma in the RPE (figure 1.4) *(Green & Enger 1993)*. Consequently neovascular AMD has a devastating effect on visual acuity.

1.3.1.5 Drusen and AMD

As mentioned previously, drusen is the main lesion associated with AMD (Sarks *et al* 1994). It represents the earliest feature of the disease that can be detected by ophthalmoscopic examination (Bressler *et al* 1988). Presence of drusen is a significant risk factor for the development of AMD (Sarks 1980, Bressler *et al* 1994). The features such as the number, size, shape and distribution of sub-RPE deposits are important characteristics that can indicate greater risk of developing AMD. It has been shown that eyes with AMD exhibit softer, more confluent and larger drusen than normal age-matched control eyes (figure 1.5) (Spraul &

Grossniklaus 1997).

Furthermore, particular types of drusen have been associated with different forms of late stage AMD and therefore may be of some prognostic importance. For instance, soft drusen have been associated with choroidal

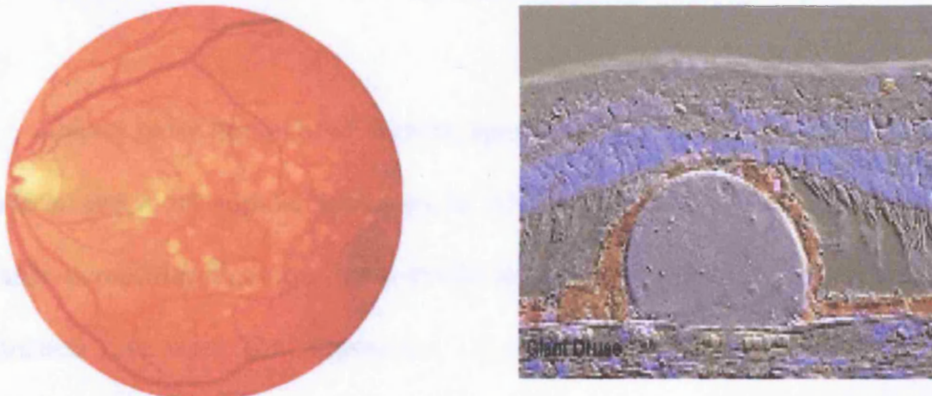


Figure1.5 The dry type is much more common and is characterized by drusen and loss of pigment in the retina. Drusen are small, yellowish deposits that form within the layers of the retina (Spraul & Grossniklaus 1997)

neovascularisation (Sarks *et al* 1994) and the presence of large or numerous drusen are significant risk factors for development of late stage AMD (Holz *et al* 1994). In fact, the Beaver Dam eye study showed that 11.7% of patients who initially had large drusen developed late stage AMD after 5 years (Klein *et al* 1997).

1.3.1.6 Risk Factors for AMD

Large numbers of studies have been undertaken by researchers to try to identify the association of the risk factors of AMD and epidemiological aetiology of AMD. The three principal research studies based on large sample populations, the Beaver Dam Eye Study (Klein *et al* 1992), the Rotterdam study (Vingerling *et al* 1995) and the Blue Mountains Eye Study (Mitchell *et al* 1995) have provided reliable epidemiological evidences to establish which risk factors are associated

with AMD. Cardiovascular disease, smoking, dietary factors, light exposure, genotype and ethnic origin have all been postulated as potential risk factors for AMD (O'Shea 1998). There is also evidence from many different studies that consistently shows that, as the name implies, age is the most important and only certain risk factor for AMD (Vingerling *et al* 1995, Mitchell *et al* 1995, Klein *et al* 1992).

Among those implied risk factors, apart from age, cardiovascular disease is considered the most popular aetiology in AMD; this implies that AMD may be primarily a vascular disorder. The vascular model proposes that choroid vascular dysfunction increases the deposition of lipid in Bruch's membrane. This mechanism is the similar to atheroma formation in the vessel wall of large vessels, which leads to deterioration of the overlying RPE (Friedman 2000).

Hypertension is considered to be one of many cardiovascular risk factors that could potentially damage the choroid blood vessels. Some other studies have found a relationship between uncontrolled blood pressure and AMD (Hyman *et al* 2000). However, none of the three large population based studies found any significant relationship between hypertension and AMD.

Another cardiovascular risk factor, atherosclerosis, has also been linked to AMD. This relationship was investigated and certified in the Rotterdam study (Vingerling *et al* 1995). The Beaver Dam Eye study found that higher levels of dietary fat intake were associated with early stage AMD (Mares-Perlman *et al* 1995).

Smoking is another identified risk factor for cardiovascular disease and it has been consistently associated with AMD. It is hypothesized that smoking might lead to an increased risk of AMD either by directly affecting the choroid

circulation or by decreasing the level of antioxidants in the blood (Evans 2001). There were numerous studies investigating the relationship between smoking and AMD; most of the results had found significant evidence linking smoking with AMD (Klein *et al* 1993, Vingerling *et al* 1996, Smith *et al* 1996). There was a lack of evidence, though, to support an association between other cardiovascular risk factors such as alcohol consumption or the effect of oestrogen with AMD.

In humans, diabetes is another cardiovascular risk but, surprisingly, it did not appear to have a significant relationship to AMD (Mitchell & Wang 1999, Klein *et al* 1992). However Bensaoula's research showed that there were morphological changes in the RPE of diabetic rats (Bensaoula & Ottlecz 2001). Further studies from this research group uncovered a significant diabetes-induced deepening of the basal infolds and an increase in the amount of intercellular space. These significant clinical alterations are not yet observed but Bensaula speculated that the changes might be related to blood retinal barrier dysfunction in diabetes (Bensaoula & Ottlecz 2001).

Furthermore, the prevalence of macular drusen was reported to be high in a population of insulin dependent diabetics (Alexander 1985). Prospective research in recent decades on the development and risk factors for AMD had shown that visual acuity in patients with AMD deteriorated earlier in type 2 diabetic patients than in non-diabetic control patients. However, the frequency of AMD was approximately the same in both groups (Voutilainen-Kaunisto *et al* 2000). An interesting finding from the same study showed that AMD was an independent risk factor for cardiovascular mortality in type 2 diabetic patients. The relative risk for cardiovascular mortality in diabetic patients with AMD was almost five times higher than in non-diabetic patients without AMD (Voutilainen-Kaunisto *et al*

2000). Therefore it is possible that AMD and cardiovascular disease have common precursors.

Further evidence to support a possible relationship between AMD and diabetes came from the discovery that some of the major pathologic features of AMD such as basal laminar deposits and choroid neovascularisation were often associated with choroid capillaries degeneration in diabetes (Cao *et al* 1998). It was also observed that the thickness of basal laminar deposits increased with increasing severity of choriocapillaris degeneration (Cao *et al* 1998). This confirms previous findings that choriocapillaris dropout and compromised choroid blood flow in diabetes was associated with basement membrane thickening (Hidayat & Fine 1985) and choroid neovascularisation (McLeod & Lutty 1994).

Exposure to light and antioxidant intake or supplementation may also be associated with an increased risk of AMD (Hawkins *et al* 1999). It is thought that over-exposure to light or abnormally low levels of antioxidants may result in higher levels of oxidative stress and as a result increase risk of AMD. A number of studies have examined the role of ultraviolet and/or visible light on the development of AMD (Taylor *et al* 1992, Cruickshanks *et al* 1993, Darzins *et al* 1997). It is extremely difficult to reliably measure exposure to light directly and therefore not surprising that no convincing evidence has been found (Evans 2001).

The role of antioxidants in AMD is equally inconclusive (Hawkins *et al* 1999) and there is little evidence that antioxidant supplementation prevents or slows the progression of AMD (Evans 2001, Taylor *et al* 2002). There are several randomised controlled trials investigating antioxidant supplementation that are in progress, and hopefully should show some future lists in the role of antioxidants in AMD.

In addition to the role of cardiovascular and environmental risk factors, there is mounting evidence to suggest that there is a genetic basis for AMD (Gorin *et al* 1999). As a result, there has been a great deal of research focused on attempting to identify the candidate genes that contribute to AMD risk. But there are difficulties in studying the role of genetics in AMD as, for example, AMD does not develop until old age. This means that large pedigrees are rare to achieve (Zack *et al* 1999). There is also a problem that AMD is likely to be a complex genetic disorder in which many genes (known and unknown) are implicated (Gorin *et al* 1999). This even further complicated by environmental factors, which may modify a genetically determined risk for AMD (Gorin *et al* 1999).

Therefore, in addition to studying the genetics of AMD directly, another approach has been to study other retinal diseases with similar phenotypes such as Startgardt's disease and Best's disease (Zack *et al* 1999). Although none of the genes that cause these diseases have been found to account for a significant proportion of AMD, these studies can be used to gain insights into the pathogenesis of this disease (Stone *et al* 2001). An important recent development to indicate a genetic component of AMD is the reduced prevalence of the ε4 allele of apolipoprotein E (also linked to atherosclerosis) in patients with exudative AMD (Souied *et al* 1998 and Klaver *et al* 1998).

In conclusion, the aetiology of AMD is complex and there are many putative risk factors. At present, age is the best-understood and only definite risk factor for AMD although there is also strong evidence to implicate smoking in the development of AMD. For the other hypothetical risk factors, there is either insufficient or conflicting evidence to support the association with AMD. Future studies could help to establish the most influential genetic and environmental

contributions to the pathogenesis and aetiology of this disease.

1.3.1.7 Current Understanding and Treatment For AMD

As outlined briefly above, the pathogenesis of AMD remains ill defined. At present there is no established treatment that can effectively restore vision in AMD. The dry form of AMD represents a slow atrophy of the cells within the macula. At present, there is no definitive treatment other than risk factor management. These methods include: 1) reducing consumption of vegetable oils; 2) stop of smoking; 3) reduction in body mass index (BMI); and 4) taking specific vitamin supplements (ie. antioxidants, omega-3) (Packo, 2004). The Age Related Eye Disease Study (AREDS) provided the best evidence for antioxidant supplementation particularly for unaffected eyes or early stage AMD (Coleman. H & Chew E 2007). However, in most instances these measures only slow the progression of the condition. Interestingly, the AREDS formulation also showed a 30% risk reduction in unaffected fellow eyes of patients with wet AMD (H Coleman & Chew E 2007).

Epidemiologic data are still clear about the fact that age-related macular degeneration (AMD) is the leading cause for substantial and irreversible vision loss among the populations of developed nations (Klein *et al.*, 1992; Mitchell *et al.*, 1995; *Vingerling et al.*, 1995). Due to the rapid aging of the western population, this number is expected to double by the year of 2020 (Friedman *et al.*, 2004). Although neovascular AMD only accounts for about 10–20% of the total AMD incidence, it is responsible for 90% of cases of severe vision loss (Votruba and Gregor, 2001). The management of neovascular AMD will therefore become an important challenge for patients, ophthalmologists and the health systems.

In recent years, research has provided new insights into the pathogenesis of macular disease. In combination with the rapidly growing knowledge about basic

mechanisms in angiogenesis, this accumulating knowledge has led to novel developments in therapeutic strategies resulting in a widening of available treatment options and improved prognostic perspectives (Ursula et al., 2007). About ten years ago, the only proven treatment for choroidal neovascularization (CNV) was laser photocoagulation applicable only in a rather small proportion of patients (C. H. Meyer 2007). Today less destructive treatments directly targeting the CNV component and its pathogenic cascade have become available.

It is the first time in the history of AMD therapy that intravitreally administered antibodies against vascular endothelial growth factor (VEGF) offer a significant chance of an increase in visual acuity to patients affected with neovascular AMD. Today, anti-angiogenic approaches can provide long-term vision maintenance in over 90% and substantial improvement in 25–40% of patients (Q. Mohamed et al., 2007). New treatment, such as RNA interference or even gene therapy hold a positive promise to improve treatment outcomes in the near future.

So far, two anti-VEGF drugs have been currently available on the market: bevacizumab (Avastin) and ranibizumab (Lucentis). Bevacizumab and ranibizumab have very similar structurre. Bevacizumab is a full size recombinant antibody molecule initially engineered and approved for the treatment of colorectal cancer (Q. Mohamed et al., 2007). As designed for systemic infusion, it has been shown to have a significant side effect profile (mainly hypertension and thromboembolic events) (Rich *et al.,* 2006). However, the minute intravitreal doses used for treating AMD, to date, have shown essentially no measurable toxicity (Avery *et al.*, 2006; Rich *et al.,* 2006)

On the contrast, Ranibizumab is derived from its parent molecule-

bevacizumab. It consists of the FAB binding fragment of the same antibody molecule targeting VEGF (Rich *et al.*, 2006). Ranibizumab is specifically designed for use in the treatment of AMD. In mouse models, FAB binding fragments have been shown to have greater retinal penetration than full size antibody molecules, presumably due to its reduced size (Mordenti *et al.*, 1999). The side effects and administering doses for ranibizumab have been studied in a large randomised controlled trial (Heier JS *et al.*, 2006). These appear to be the only theoretical advantages of one over the other. Both drugs seem to show similar clinical effects when using optical coherence tomography (OCT) images as a guide to treatment responses.

There are also some other new drugs undergoing evaluation all around the world. These include pegaptanib (Macugen) and anecortave acetate (Retaane). Pegaptanib is another agent directed at inhibiting the specific isoform 165 of VEGF (Bird M *et al.*, 2007). Its use as an independent agent is confounded by the fact that the majority of initial study eyes had already been treated with verteporfin PDT. The studies did not seem to show the same degree of visual improvements seen with drugs such as ranibizumab (Gragoudas *et al.* 2004).

Anecortave acetate is an angiostatic cortisene compound designed to block the migration of proliferating endothelial cells by inhibiting metalloproteinases. Phase III randomised, double masked data showed anecortave acetate to be no better than standard verteporfin PDT (Slakter *et al.*, 2006). Neither pegaptanib nor anecortave acetate is set to be marketed in the world. They are undergoing further trial to become available for clinical application.

1.4 AGEs/ALEs and Tissue Dysfunction

1.4.1 What Are AGEs/ALEs

Advanced Glycation end products are forms of heterogeneous group of irreversible adducts comes from reactive derivatives of nonenzymatic glucose-protein condensation reactions, as well as lipids and nucleic acids exposed to reducing sugars. They were originally characterized by their yellow-brown fluorescent colour and their ability to form crosslinks between amino groups (Vlassara et at., 1984). It is now used for a broad range of advanced products of the Maillard Reaction (Njorge et al., 1989). The compounds include such as (3,4,)N^{ε}*-(carboxymethyl)* lysine(CML) and pyrraline, which neither show colour and fluorescence, nor occur as cross-links in proteins (Reddy et al., 1995). The formation of AGEs *in vitro* and *in vivo* are both dependent on the turnover rate of the chemically modified target, time and sugar concentration.

1.4.2 Risk Factors

1.4.2.1 Age

Advanced glycation end products are a subgroup of the non-enzymatically derived crosslinks. They are the latest products that modify proteins and nucleic acids by reducing sugar, and they have been shown to accumulate slowly in Bruch's membrane as well as accumulating at renal and vascular smooth cells, which progressively increases in thickness throughout life and at a more rapid rate in diabetes (Li et al., 1996).

Such findings have contributed to the prevalent view that random

modifications of protein molecules cause a generalized decrease in metabolic efficiency occurring during senescence. In some subjects, this may contribute to choroidal neovascularization, or to geographic atrophy with consequent visual loss (Marmor & Wolfensberger, 1998).

1.4.2.2 Diet

Recent studies show that AGEs/ALEs can be introduced to biological systems from exogenous sources. This may have significant impact on disease mechanisms. Diet is the major source of exogenous AGEs/ALEs, with the highest content in complex foods, such as those rich in carbohydrates and fats (Vlassara. *et al.,* 2002). Formation of AGEs/ALEs is enhanced by exposure to heat; this is responsible for the browning of food as it is cooked, increasing with cooking temperature and duration.

Koschinsky and colleagues (1997) showed that AGE immuno-reactivity could increase by 200-fold in egg-white cooked with fructose compared with egg-white prepared identically in the absence of fructose. This study also confirmed the absorption of 10% of ingested AGE and showed that only one-third of that absorbed was excreted within 48 hours in the urine of patients with normal renal function. The AGEs that are not cleared by the kidney were distributed to the tissues where they remained biologically active. This data implies that dietary AGEs may pose a significant environment risk, particularly to patients with nephropathy (Kischinsky *et al.,* 1997).

1.4.2.3 Smoking

Smokers have a significantly higher risk for developing coronary and cerebrovascular disease (Carla, *et al.,* 1997). As tobacco leaves are dried in the

presence of sugar, a process called curing (Vlassara & Palace, 2000); the Maillard reaction causes the formation of glycated and oxidized derivatives. After combustion, reactive AGE species are volatilized (Vlassara &Palace 2000). The species, also termed 'glycotoxins', react with protein, exhibit a specific fluorescence when cross-linked to protein and are mutagenic are inhaled, absorbed through the lungs and become conjugated with serum proteins including lipoproteins.

This was reflected in the fact that total serum AGE and AGE-apoprotein β levels in cigarette smokers were found to be significantly higher than in non-smokers. This suggests that increased glycotoxin exposure might contribute to the increased incidence of atherosclerosis, lens optical density (Hammond, 1999) and ocular disease (Cheng, *et al.*, 2000)

1.4.2.4 Alcohol/Ethanol

Epidemiological studies suggested that there was a beneficial effect of moderate ethanol consumption on the incidence of cardiovascular disease (Yousef *et al.,* 1998). Upon ingestion, alcohol/ethanol is oxidatively metabolised by alcohol dehydrogenase to produce acetaldehyde. It can react with nucleotrophes to form covalent additional products, which is chemically reactive. Its activity in modifying macromolecules has been hypothesised to contribute to certain organ toxicities associated with chronic ethanol abuse (Yousef *et al.,* 1998).

Yousef and colleagues identified a biochemical modification produced by the reaction of acetaldehyde with protein-bound Amadori products. Amadori products typically arise from the nonenzymatic addition of reducing sugar to protein amino groups and are the precursors to irreversibly bound, cross-linking

moieties (AGEs).

1.4.2.5 Protein Glycation

The nonenzymatic reaction of the amino groups of amino acids, peptides and proteins with reducing sugars to form a complex family of rearranged and dehydrated covalent adducts, that are often seen as yellow-brown and/or fluorescent and include many cross-linked structures, was first studied under defined conditions by L.C. Maillard in the early 1990's It is termed the Maillard reaction or advanced glycation (Ulrich, *et al.,* 2001).

Food chemists have long studied the process as a source of flavour, colour, and texture changes in cooked, processed, and stored foods. During 1970's and 1980's, it was realised that this process also occured slowly *in vivo*. AGEs are implicated in causing the complications of diabetes and ageing, primarily via adventitious and cross-linking of protein (Morgan, *et al,* 2002)

1.4.2.6 Oxidative processes

The ageing process or senescence is characterised by a ubiquitous decline in the functional capacity of various physiological systems. It is perhaps most noticeably manifested in the loss of motor ability and the stamina for sustained physical effort (Head, 2002). Ageing is a consequence of the accumulation of random oxidative damage to cellular molecules, caused by reactive oxygen or nitrogen species, produced under normal physiological condition.

Many studies have found that the oxidative modifications clearly affect nuclear and mitochondria DNA, they are also readily observed in proteins (Liang, *et al.* 1997). Oxidative modified proteins are generally dysfunctional, losing catalytic or structural integrity. Thus, oxidative damage to protein is considered to

be a key of importance in ageing. A linear relationship between subject age and the amount of protein carbonyl groups has been found in the human eyes lens cortex by Fancesco (2000).

1.4.3 Chemical Background of Glycation and AGEs/ALEs Under Physiological Conditions

1.4.3.1 Biochemistry of AGE Formation

1.4.3.1.1 The Maillard Reaction-the source of endogenous AGEs/ALEs

AGEs are formed by a complex series of nonenzymatic glycation reactions. AGE formation is initiated by the nucleophilic addition of a reducing sugar such as glucose to a free amino group. The free amino group could be the N-terminal amino group of a protein or the ε-amino group of the amino acids lysine or arginine, Although glycation reactions can also occur on free amine containing lipids and on DNA. In any case, this reaction results in an unstable Schiff base, which undergoes rearrangement to form a more stable Amadori product.

Amadori product formation is also known as glycation. Therefore, any protein which attached by Amadori Products is characterised as a glycated protein. It is important to notice that this is not as the same as an enzymatically glycosylated protein (Ulrich & Cerami 2001. Early glycation products, such as

hours ⟶ *days* ⟶ *weeks / months*

GLUCOSE + NH_2 - R ⇌ SCHIFF BASE ⇌ AMADORI PRODUCT ⟶ ADVANCED GLYCATION ENDPRODUCTS

H, O, C, $(CHOH)_4$ + NH_2-R ⇌ H, NH-R (+), C, $(CHOH)_4$, CH_2OH ⇌ CH_2NH-R, C=O, $(CHOH)_3$, CH_2OH ⟶ AFGP; CML; PYRRALINE; FFI; PENTOSIDINE

Figure 1.6 Formation of protein-bound nonenzymatic glycosylation products Glucose (in the open-chain form) reacts with amino groups on proteins, lipids or DNA to form unstable intermediates such as Schiff bases and Amadori products, which are freely reversible and exist in equilibrium. Further condensation and oxidation reactions lead to formation of AGEs, which are irreversible and are formed over a longer time (H.Vlassara & M.R. Palace 2002).

Schiff bases and Amadori-products are chemically reversible. They are in equilibrium with glucose, and are fairly stable and have half-lives of days and weeks (Figure 1.6). These properties allow the level of glucose derived Amadori products to be a good indicator of blood glucose control over a period of weeks. Hence it is used as the routine clinical measurement of HbA1c levels of diabetic patients (Koenig *et al* 1976).

But, as Amadori products exist in equilibrium, after they have reached a steady state, they do not increase over time (Vishwanath *et al* 1986). That means the levels of Amadori products in diabetic patients are no more than 2-3 fold higher than in non-diabetic patients (Kato *et al* 1989). Consequently, it has been suggested that it would be more reliable to measure AGE-modified haemoglobin as it is irreversible and reflects glucose control over a longer period of time (Makita *et al* 1992).

Consequently, following the Amadori rearrangement the product can either

undergo further reactions to form advanced glycation end products or it can be broken down to regenerate the intact amine, in which case the sugar component goes on to form highly reactive dicarbonyl compounds, for example like glyoxal, methylglyoxal or deoxyglucosones (Thornalley *et al* 1999). These compounds are intermediates that propagate the Maillard reaction. They are an important source of AGEs because they form an alternative pathway that leads to the formation of many AGE structures (Thornalley *et al* 1999).

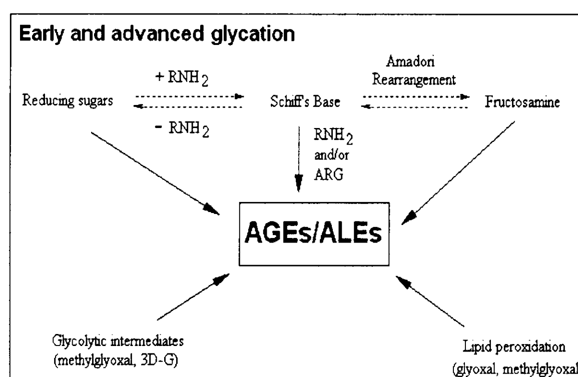


Figure 1.7 Diagram of Formation of AGEs: AGEs and the related advanced lipoxidation endproducts (ALEs) can occur through a number of inter-related pathways, some of which can lead to very rapid formation of these adducts. (A. W. Stitt 2003)

The Maillard reaction processes give rise to these AGEs is not well understood. Although it is known that following the Amadori rearrangement, further dehydration, cross linking and condensation reactions occur and result in the formation of advanced glycation end products. These products are thermodynamically stable, and thereby irreversible and permanent. For this reason, they accumulate over time (Figure 1.7). On account of these properties, AGEs are predisposed to accumulate on proteins with long half-lives and are capable of forming covalent bonds with amino groups on other proteins (A.W. Stitt et *al* 2003).

The formation of AGEs is proportional to time and sugar concentrations.

This means that AGEs may form very quickly if reactive sugars or intermediates are at high levels (Thornalley *et al* 1999). Therefore, the hyperglycaemia in diabetes results an accelerated rate of formation of AGEs. However, it is also important to know that advanced glycosylation reactions occur with ageing even under normal blood glucose levels, which results in an increasing of AGEs' levels with ageing.

1.4.3.1.2 Major Exogenous Sources of AGEs

Recent studies suggest that AGEs introduced to biological systems from exogenous sources, such as diet and smoking may have significant impact on disease mechanisms. Diet is the major source of exogenous AGE with the highest content in complex foods, such as those rich in carbohydrates and fats. The formation of AGE is enhanced by exposure to heat; thus AGE content, which is responsible for the browning of food as it cooks, increases with cooking temperature and duration. Whilst the abundance of dietary AGEs including methylglyoxal (MG) and Carboxymethyl lysine (CML) has long been recognized. Their significance as potential toxins was not appreciated as their absorption was estimated to be only approximately 10% of that ingested (Stitt 2001).

Smoking seems to be another source of orally administered exogenous AGEs, since the level of AGE modified proteins in the serum of cigarette smokers is significantly higher than in non-smokers (cerami et al 1997); also significantly increased serum AGE levels have been observed in diabetic smokers compared to diabetic non-smokers (Baurngartl *et al.,* 1996). It is reasonable to say that breaking the course of the AGE can be supported by an antioxidant diet and a healthy life

style.

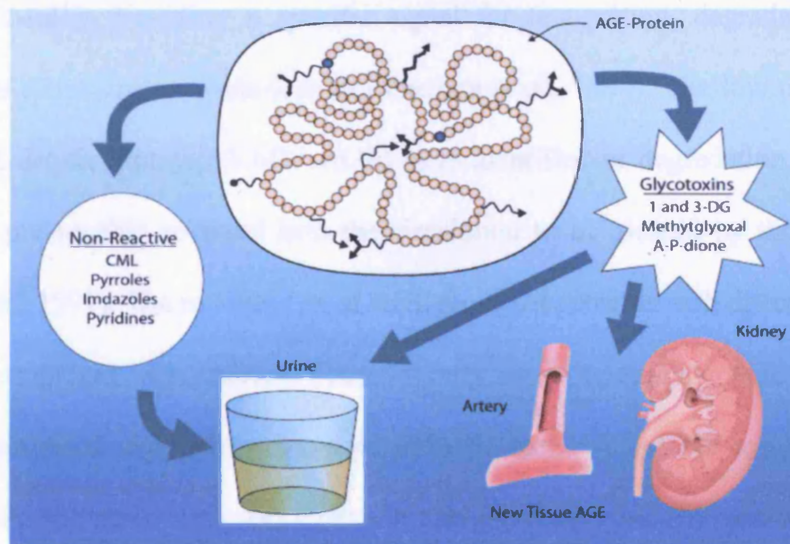


Figure 1.8 Schematic representation of the fate of diet-derived AGEs. Cooked foods contain sugar-derived protein or lipid glycation intermediates that may include noncrosslinking products, such as N-ε-carboxymethyl-lysine (CML), pyrroles, imidazoles, pyridines (left insert), or crosslink forming, reactive intermediates (glycotoxins), such as 1-,3-deoxyglycozone (3-DG), methylglyoxal, protein-linked A-P-dione (right insert). The former are presumably readily excreted in urine, whilst the latter may reattach onto serum or tissue components to form new AGEs with the eventual pathological consequences. (Vlassara, et al., 2002)

It has been found that food AGEs can be absorbed after eating (figure 1.8). In Koschinsky's research in 1997, food-derived AGEs with cross-linking activity could accumulate in serum to a significant degree, implying that normal kidney function is important to protect from dietary AGEs.

Further *in vivo* evidence comes from studies on hyper-cholesterol mice, which found that mice fed a low-AGE diet had a reduced vessel wall response to arterial injury, indicating that dietary AGEs may enhance susceptibility of various tissues to disease (Lin *et al* 2002). These studies indicate that exogenous sources of AGEs may be important pathways for many disease processes.

1.4.4 Biopathogenesis Relation to AGEs/ALEs Formation

1.4.4.1 The Cellular Action of AGEs

AGE formation was originally thought to specifically tag senescent proteins, thereby providing a specific signal for recognition, degradation and removal of senescent macromolecules (Vlassara *et al.,* 1994). The low molecular weight AGE-rich peptides (LMW-AGE) were identified as degradation products and were presumably released into the circulation to be cleared by the kidneys (Yang *et al.,* 1991). The interactions of AGE modified proteins with different AGE receptor complexes were studied. They not only served to degrade AGE-proteins, but also activated signal transduction pathways that induced the synthesis and released of cytokines and growth factors that might initiate tissue repair and protein turnover (Vlassara *et at.,* 1994).

Intracellular AGE formation occurs extremely fast because intracellular sugars are much more reactive than glucose. Giardino reported in 1994 that 1 week *in vitro* and *in vivo* incubation of endothelial cells in the presence of high glucose levels resulted in a 13.8-fold increase in the intracellular AGE content. In parallel, the mitogenic activity of high glucose cultivated endothelial cells markedly decreased. The observed loss in mitogenic activity was due to post-translational modifications of basic fibroblast growth factor (BFGF) by AGEs, representing the major AGE-modified protein in endothelial cells (Giardino et al., 1994).

The extracellular matrix AGE formation results in decreased elasticity; increased thickness; rigidity; breaking time and narrowing of the vessel lumen (Bierhaus et at., 1997). Binding of AGEs to their cellular binding sites resulted in depletion of cellular antioxidant defence mechanisms and the generation of oxygen free radicals.

1.4.4.2 AGE-Induced DNA Rearrangement

Lee and Cerami reported in 1990 that AGEs could form on DNA, particularly on guanine residues to induce unusual DNA rearrangements. The strains of Є.coli that accumulate high levels of glucose-6-phosphate, is particularly active in forming AGEs (Lee and Cerami, 1991). Some evidences have also been found in mammalian cells that AGE formation on DNA may be responsible for insertions containing repetitive sequences of the Alu family that have been found to disrupt human genes (Bucala *et al.,* 1993a). It has important implications that the possibility of AGEs may induce genetic rearrangements *in vivo*; for example, as a possible cause of congenital malformations in infants of poorly controlled, insulin-dependent, diabetic mothers (Lee *et al.,* 1995)

1.4.4.2.1 AGEs Involved in Ocular Diseases

There are a large number of evidences showed that AGEs were also involved in the pathogenesis of ocular diseases. Many of the cells and tissues of the eye are severely affected by diabetes and ageing, thereby non-enzymatic glycation has been implicated as an important process in several different ocular diseases (Stitt 2001).

The role of non-enzymatic glycation in cataract formation is the most widely researched. This is because the fact that lens crystallins are proteins. They have very long half-lives and have been used extensively for studying the effects of non-enzymatic glycation *in vivo* (Stitt 2001). There was a substantial amount of evidence to show that there were increased amounts of AGEs in aged and diabetic lenses (Harding & Crabbe 1984, Stevens *et al* 1978, Matsumoto *et al* 1997, Stitt 2001). Levels of AGE crosslinking were high in aged and cataractous lenses with brunescent lenses (Chellen *et al* 1999). AGEs contributed to cataract formation by

crosslinking the lens proteins leading to aggregate formation (Chellan *et al* 1999, Shamsi *et al* 2000).

AGEs may also be involved in modifications of the cornea in diabetes (Sady *et al* 1995, Kaji *et al* 2000) and ageing (Malik *et al* 1992, Malik & Meek 1994). AGEs have been immunologically identified in the corneal epithelial basement membrane, which is heavily glycated in diabetics (Kaji *et al* 2000). An *in vitro* model of glycated basement membrane has shown that nonenzymatic glycation of laminin on a culture dish can decrease adhesion and spreading of corneal epithelial cells (Kaji *et al* 2000). Furthermore, human corneal collagen contains significant AGE levels that increase with ageing and are accompanied by structural changes such as increased cross-linking and increased collagen intermolecular spacing (Malik *et al* 1992). Many of these structural changes can be inhibited by inhibitors of protein glycation such as aspirin-like compounds and certain vitamins (Malik & Meek 1994, 1996). Advanced glycation has also been implicated in the development of vitreous pathology (Sebag *et al* 1992). Vitreous AGE levels have been shown to increase with age in non-diabetics and in diabetics (Stitt *et al* 1998). Similar to the corneal situation, the collagen component of the vitreous gel is also subject to modification by advanced glycation. This process can also be prevented by the use of an AGE inhibitor (Stitt *et al* 1998). It appears that this non-enzymatic glycation of collagen causes dissociation from hyaluronan and consequently causes destabilisation of the complex vitreous gel structure (Stitt 1998).

AGEs are also thought to have significant effects on the retinal vasculature. AGEs have been localised to the retinal vessels in diabetes and so may have a role in the development of diabetic retinopathy (Stitt *et al* 1998). Levels of the defined

AGE adduct CML have been correlated with diabetic retinopathy (Stitt 1997, Ono *et al.,* 1998, Chiarelli *et al.,* 1999). Similarly increased levels of pentosidine in skin also correlate with severity of diabetic retinopathy (Chiarelli *et al.,* 1999). A number of studies have shown that AGEs can induce wide-ranging effects on retinal cells. Nitric oxide synthase expression by retinal endothelial cells is inhibited by AGEs (Chakravarthy *et al* 1998).

AGEs can also cause calcification of retinal pericytes (Yamagishi *et al* 1999) and can modulate cell growth (Ruggeriero-Lopez *et al* 1997, Chibber *et al* 1997). At low concentrations they can be mitogenic to retinal endothelial cells increasing the cell proliferation, while at higher concentrations, they can have a toxic effect (Chibber *et al* 1997). AGEs can stimulate cell proliferation through the induction of retinal vascular endothelial grow factor (VEGF) (Lu *et al* 1998, Hirata *et al* 1997). VEGF is also required for angiogenesis and so AGEs are thought to participate in the pathogenesis of diabetic retinopathy through their ability to increase retinal VEGF expression (Yamaguishi *et al* 1997). Finally, as stated previously, there is a growing amount of evidence to associate the accumulation of AGEs with age-related dysfunction of the RPE and Bruch's membrane, which may be important in the pathogenesis of AMD.

1.4.4.2.2 AGEs/ALEs in RPE Ageing

The relationship of AGEs and ageing is based on the observation that reactive AGE adducts accumulate on the extracellular matrix and in the intracellular compartment of target cells (Stitt & Vlassara, 1999). By the Maillard reaction, AGEs can form on the amino groups of proteins, lipids and DNA and also by reactive dicarbonyls formed via auto oxidation of sugars, lipid oxidation and many metabolic pathways' by-products (Baynes et al., 2000). ALEs are the

protein-lipid peroxidation and dicarbonyl reaction products. They commonly form in concert with AGEs. Their pathogenic role in ageing has a strong links between oxidative and glycation reactions.

Odetti et al., (1998) have identified that AGEs/ALEs accumulated in lipid-rich CNS cells with ageing. Because the RPE-resides in a highly oxidative environment and constantly phagocytoses lipid/protein rich photoreceptor membranes, it would be expected that AGEs and ALEs would accumulate in large amounts in both the intra- and extracellular compartments of the RPE with increasing age.

1.5 RESEARCH AIMS & OBJECTIVES

The evidence suggests that accumulation of advanced glycation end products in combination with advanced lipoxidation end products with ageing may play a significant, pathogenic role at the RPE-Bruch's axis. Therefore we hypothesise that AGEs/ALEs may have a critical role in age-related dysfunction of the RPE. In order to address our hypothesise, our main objectives are:

1. To investigate AGEs/ALEs accumulation in ageing RPE cells
2. To identify the effects of AGE-modified sub-cellular matrix on RPE cell function
3. To study the pathgocytic and degradative capacity of RPE exposed to AGEs/ALEs.
4. To evaluate the kinetics of key lysosomal proteinases in RPE cells after AGE/ALE-exposure and the correlation with lipofuscin accumulation.
5. Gene expressions of the key lysosomal proteinases will be analysed before and after cells have glycation treatment.

Chapter 2. In Vitro Assay to Identify the Effects of AGEs/ALEs on Lipofuscin Accumulation in RPE Cells;

2.1 Introduction

Current research has found that the most important function of the RPE is the phagocytosis and degradation of photoreceptor outer segments, which is essential to maintain healthy photoreceptor-cell function. It has been estimated that a single RPE cell can phagocytose and degrade approximately 2000 – 4000 outer segment membranous discs each day (Bok & Young 1969). This impressive workload for the RPE may also play a significant role in many age-related diseases in the retina. In fact, a large amount of evidence suggested that the lysosomal system of the RPE could suffer significantly during the ageing process and be subject to dysfunction. This may be presented by decreased degradation of POS, increased lipofuscin deposition and drusen formation in the sub-RPE space (Hayasaka 1983).

Research by Feeney L (1978) and Boulton M (1991) have demonstrated that lipofuscin is a lipid-protein aggregate that was present within the human retinal pigment epithelium as yellow-brown refractile granules showing a characteristic fluorescence under short wavelength light. Lipofuscin granules are thought to represent the lifelong accumulation of lysosomal residual bodies containing the end products of phagocytosis of POS (Katz Ml et al., 1986) and, to a lesser extent, of autophagy (Feeney L 1978). The intracellular accumulation of these granules, which can constitute as much as 19% of RPE cytoplasmic volume

in later life (Feeney-Burns L et al., 1984), has been implicated in the development of age-related macular degeneration (Boulton M 1991).

There is also increasing evidence to suggest that AGEs may have an important role in age-related RPE dysfunction. So far, AGEs have been identified and known to be endocytosed by many cell-types, after which they are directed to secondary lysosomes (Mori *et al* 1995, Araki *et al* 1995; A.W. Stitt *et al* 2000) and it has been shown that AGEs accumulate in the lysosomal compartment in other non-retinal cell types (Figure 2.1).

Numerous studies, both in *vitro* (Boulton et al., 1989; Rakoczy et al., 1992; Burke and Brzeski-Skumatz, 1996) and *in vivo* (Eldred and Katz, 1988; Katz and Nornberg, 1992), have investigated the mechanisms involved in the formation of RPE lipofuscin. These studies assessed the accumulation of cellular autofluorescent granules and interpreted this to be indicative of lipofuscin formation.

In this chapter, the contribution of photoreceptor outer segments and autophagy to granule formation was assessed by 1) feeding RPE cells with POS (vary concentrations) and in long-term RPE cell culture with and without glycated condition (vary glycation conditions). The degree of the protein and lipid components of these substrates in granule formation was determined by flow cytometer.

2.2 Materials

ARPE-19 cells were obtained from stocks held in Professor Mike Boulton's lab. All human eyes came from the Bristol eye bank. Chemicals were purchased from the suppliers listed in Appendix II.

2.3 Methods

2.3.1 Primary RPE And ARPE-19 cell Culture

2.3.1.1 Primary RPE cell culture

All human eyes (which received full permission for research use according to UKTS guidelines) were collected from the Bristol eye bank. The donors used in this study were a 58 year old male, a 69 year old male and a 73 year old female. All eyes were enucleated less than 48 hours post-mortem as RPE cells could only be cultured from eyes that had been enucleated and dissected less than 48 hours post-mortem. After prior removal of the corneas for transplantation at Bristol eye bank, under sterile conditions in a laminar flow cabinet, eyeballs were circumferentially dissected approximately 2mm posterior to the limbus and the anterior portion of the eyes was discarded.

The process was that each of the remaining eyecups was placed in a sterile eye-cup-holder after gentle removal of the lens, vitreous and the attached neural retinal. The RPE layer was then exposed to 1 ml of 0.25% trypsin-EDTA (appendix II) and incubated at 37°C for 40 minutes in a 60ml polyethylene pot. The RPE cells were detached by gentle agitation using a 3ml plastic Pasteur pipette. The resultant cell suspension was added to 5ml serum-containing growth medium (Ham's F10 nutrient mixture supplemented with 10% FCS + 1%

antibiotics and fungizone) (appendix II) to inactivate the trypsin-EDTA.

The cell suspension was then split, according to pellet size, between a numbers of wells in a 24-well plate. Plates were then incubated at 37°C in a CO_2 incubator (95% air, 5% CO2) for approximately 7 days to allow attachment. The growth medium was subsequently replaced with fresh growth medium every three or four days and the RPE cells were regularly observed through an inverted microscope until the cells reached 70% confluence. After confluence, cells were detached by Trypsin-EDTA and sub-cultured in 25 cm^2 cell culture flask for experiment. Primary RPE cell cultures became passage 1 after splitting. The purity of the primary RPE cells was confirmed by immunostaining using pancytokeratin.

2.3.1.2 ARPE-19 cell Culture

ARPE-19 cells, which was from a 19 year old male donor, were obtained from an established, spontaneously transformed cell line (ATCC number: CRL-23027), through selective trypsinisation of a primary RPE culture resulting in a uniform population of highly epithelial cells exhibiting strong growth potential (Dunn et al., 1996). It is an immortalised cell line which has transformed and altered growth properties. The cells are no longer divided because of DNA damage or shortened telomeres. This means that it will not enter replicative senescence - ie that it will not stop growing even when it is kept passing it and is viable after a large number of passages. As ARPE-19 cells came from the primary cell culture, it has the similar structural and functional properties as RPE cells *in vivo*, for example, it keeps the similar phagocytosis ability of POS, so that they can be used as model for *in vitro* studies of RPE physiology.

The same growth medium was used as the primary RPE cultures and the

same incubation conditions. More precisely, the growth medium from the cells (in 75cm^2 flasks) of confluent RPE cell cultures was aspirated and then the cells were washed with PBS. Then the cells were detached by the addition of 3ml trypsin-EDTA and incubated at 37°C for approximately 2 mins. The detachment of the cells was monitored under an inverted microscope. The trypsin-EDTA was then inactivated by adding 3ml of growth medium to re-suspend the cells. The suspension was then split between flasks (at a ratio of 1:3) and thus, the cell numbers were amplified. The ARPE-19 cells at passage 1, for example, then became passage 2. The purity of the ARPE-19 cells was confirmed by ATCC.

When primary RPE cell cultures and ARPE-19 cell cultures reached confluence, they were trypsinised again and either a) centrifugation at 100g for 5 mins at 4°C after washing with PBS, the cell pellet was re-suspended and stored in 10% FCS +1%DMSO containing growth medium in liquid nitrogen as passage 2 cells (the cells were gradually brought down to sub-zero temperatures using a container filled with isopropanol and stored at -80°C for 24hours before storage in liquid nitrogen); or b) split into 6-well plates using 10% FCS + 1% antibiotics containing growth medium for experimental purposes.

2.3.2 Isolation of Photoreceptor Outer Segments (POS)

POS were isolated by the method described by Papermaster (1982) from light-adapted bovine eyes obtained from a local abattoir. The anterior portion of the eye was removed by circumferential incision and the vitreous was discarded. By detaching from the optic nerve, the retina was collected in a cryovial. A total of 50 retinas were collected. All the collected retinas were then

frozen at -20°C for later use.

Frozen retinas were thawed at RT and 10ml retina homogenising medium (appendix II) was added per 7 retinas. The retinas were then homogenised using a handheld homogeniser and the resultant homogenate was centrifuged at 1900g for 4 minutes at 4°C (Burkard Koolspin centrifuge) producing a pellet of cellular debris. The supernatant was then collected and diluted to three times its original volume with 0.01M tris-acetate buffer, pH 7.4, and centrifuged at 1900g for 4 minutes to produce a pellet of crude POS. This pellet was re-suspended in 0.5ml of a 1.10g/ml sucrose solution containing 50μl) each of 0.1M $MgCl_2$ and 1.0M tris-acetate buffer.

A discontinuous sucrose gradient was then made using 6ml each of a 1.11, 1.13, and 1.15g/ml sucrose solution (Appendix II). The previous suspended pellet of crude POS was laid onto the top of the gradient and centrifuged at 103,000g for 30mins at 10°C. After centrifugation, the POS which appeared as an opaque white band located between the 1.11 and 1.13g/ml interface were removed using a sterile syringe and washed 3 times by PBS 1900g for 5minutes at 4°C. After that, the POS were re-suspended in growth medium with 2% FCS + 1% antibiotics and fungizone and stored at -20°C until use.

The number of POS/ml was determined using a haem ocytometer. Duplicate samples were counted from each isolation.

2.3.3 Analysis of Autofluorescent Granules Generated in Cultured RPE cells (Primary and ARPE-19) Following Challenge with POS

2.3.3.1 Sample preparation

Table2.1 The plan of feeding RPE cells with POS in different time point and conditions

day 1	Medium	Medium +POS $1X10^6$	Medium +POS $1X10^7$
day 7	Medium	Medium +POS $1X10^6$	Medium +POS $1X10^7$
day 14	Medium	Medium +POS $1X10^6$	Medium +POS $1X10^7$
day 28	Medium	Medium +POS $1X10^6$	Medium +POS $1X10^7$

ARPE-19 and primary cell cultures were carried out by culturing at 37°C in T75cm^2 flasks in the absence of bright light. Materials were prepared under standard laboratory lighting (fluorescent strip lights) and all experiment were repeated at least three times.

The confluent (80-90% confluence) Primary RPE (P1) and APRE-19 cells were detached by 5ml of 0.25% trypsin-EDTA in T75cm^2 culture flasks and then 5 ml of Ham's F10 Medium (10% FCS+1% antibiotics and fungezone) was added to each flask to neutralise the trypsin-EDTA. After centrifugation at 4°C of 100g to remove the trypsin- EDTA, cells were resuspended by 150 ml (T75 flask) of Ham's F10 Medium (10% FCS+1% antibiotics and fungezone). Then, 3ml of the mixture of cells was transferred into 6-well plates containing Ham's F10 Medium (10% FCS+1% antibiotics and fungezone). The medium was changed every 3-4 days until the cells reached confluence; then the culture medium was changed to Ham's F10 medium with 2% FCS + 1% antibiotics and fungizone for 24hours. Thereafter, the medium with 2% FCS+1% antibiotics and fungizone were used for each experimental procedure.

2.3.3.2 Challenge of RPE cells with POS (Primary and ARPE-19)

POS were diluted with Ham F10 medium (2% FCS+1% antibiotics) from stock concentration to $1x10^8$/ml and $1x10^9$/ml. For a negative control, only 4ml of culture medium was added in each well. For $1x10^6$/ml concentration treatment of

POS, 3.96ml of medium and 40μl of $1x10^8$/ml POS were added in each well (2 wells). For $1x10^7$/ml concentration treatment of POS, 3.96ml of medium and 40μl of $1x10^9$/ml POS were added in each well (2 wells). Medium with and without POS was changed every 2 days until the end time point of the experiment (i.e. day 1, 7, 14, 28).

2.3.3.3 Flow Cytometry

The mean autofluorescence per population of RPE cells was determined by using a fluorophotometric flow cytometer (Benton Dickson FACS analyser), based on the method of Rakoczy et al. (1992). Basically, the generation of autofluorescent granules of 10,000 RPE cells was measured using an excitation wavelength of 488nm, monitoring emission wavelength at 530nm. Measurements were taken less than 1 hour after cells were harvested and the data was analysed by using Cell Quest Software and single histogram statistics. The cell population to be analysed was selected by gating, using the samples trypsinised at Day=0; only samples selected within the same gate were compared.

After ARPE-19 and primary RPE cell culture had reached the experiment time points, the culture medium was removed by aspiration and then 200μl of Trypsin-EDTA were added to each well and incubated in a 37°C incubator for about 2 minutes to detach the cells. After that, 2ml of PBS with Ca^{+2} and Mg^{+2} were added to re-suspend the cells which were then transferred into a 10ml glass centrifuge tube and centrifuged at 250g for 4 minutes at 4°C. The supernatant was gently poured out until about 500μl of PBS was left. The PBS was mixed with cells gently which were then analysed for the accumulation of auto fluorescent granules by flow cytometer

2.3.3.4 Light/Fluorescence microscopy and photography

Samples prepared as described in section 2.2.3.1 were photographed at each experiment time point under both light and fluorescence (blue light) microscopes.

2.3.4 Analysis of Auto-fluorescent Granules Generated in RPE Cells (Primary and ARPE-19) Cultured on Advanced Glycated Extracellular Matrix fed with POS

2.3.4.1 Preparation of advanced glycated extracellular matrix

AGE-modified subcellular matrix (AGE-matrix) was prepared as described by Kuyuza et al (1998). The frozen reconstituted basement membrane extract matrigel (Becton Dickenson) was defrosted overnight on ice in a fridge at 4°C and diluted 1:10 with Ham's F10 basal cell culture medium. Aliquots of 0.5ml diluted matrigel were added to wells in a 6-well plate and allowed to polymerise for 1 hour at 37°C in the cell culture incubator (95% air + 5% CO_2).

Glycation of matrigel was achieved through exposure of the matrigel to glycoaldehyde (Sigama-Aldrich Co., Ltd. UK) (50, 100, 150 and 200 mM in PBS) for 4 hours at 37°C in the incubator. For a negative control, only PBS was added to the well. The glycation reaction was stopped by adding 50mM final concentration of Sodium Bromide to each well at 37°C for 2 hours. The glycated matrigel was then rinsed twice with PBS to remove un-reacted glycoaldehyde and bubbles. Gels were then ready for cell culture experiments.

2.3.4.2 Samples preparation

Table2.2 The plan of RPE cells cultured on glycated matrigel feeding with POS with different concentrations and time scales

	MGEL ONLY	50mMGA	100mMGA	150mMGA	200mMGA
day 1	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$
day 7	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$
day 14	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$
day 28	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$	$1X10^6$ and $1X10^7$

Briefly, confluent Primary (P2) and APRE-19 cells (P2) were detached using 5 ml of trypsin-EDTA in $T75cm^2$ culture flasks and incubated in the 37^oC incubator with 5% CO_2 for 2 minutes. Then, 5 ml of Ham's F10 Medium (10% FCS+1% antibiotics and fungizone) was added to each of flask to neutralise the trypsin-EDTA. After centrifugation at 4^oC of 100g to remove the trypsin- EDTA, cells were resuspended by 150 ml (each of $T75cm^2$ flask) of Ham's F10 Medium (10% FCS+1% antibiotics and fungizone). Then 3ml of the mixture of cells was transferred into the 6-well glycated matrigel plates. Ham's F10 Medium (10% FCS+1% antibiotics and fungezone) was changed every 3rd day until the cells reached confluence. Then the culture medium was changed to Ham's F10 medium with 2% FCS + 1% antibiotics and fungizone for 24hours. Thereafter, the medium with 2% FCS+1% antibiotics and fungizone were used until the end of each experimental time point.

RPE cells were challenged with POS as described in section 2.3.3.2. Culture medium with and without POS were changed every 48 hours until the end time point of the experiments (day 1, 7, 14, 28).

2.3.4.3 Flow Cytometry

Parameter setting of FACS was the same as in section 2.3.3.3. After ARPE-19 and primary RPE cultures reached the appropriate experimental points, the culture medium was removed by aspiration and then 200μl of Trypsin-EDTA were added in each well and then incubated in a 37°C incubator for about 2 minutes to detach the cells. Afterwards, each of 2ml of PBS (with Ca^{+2} and Mg^{+2}) was added to re-suspend the cells which were then transferred into a 10ml glass centrifuge tube for centrifugation at 250g for 4 minutes at 4°C. The supernatant was gently poured out until about 500μl of PBS was left. The PBS was mixed with cells gently and then auto-fluorescent granule accumulation generated by feeding POS to RPE cells (Primary and ARPE-19) was analysed by flow cytometry.

2.3.4.4 Light/Fluorescence microscopy and photography

Samples preparations for microscopy were as described in section 2.3.3.4. At each time point, photographs were taken under both a light and fluorescece Microscope (blue light).

2.4 Results

2.4.1 Light/Fluorescent Microscopy and Photography

2.4.1.1 Primary Cell Isolation and Culture

The primary RPE cells isolated from fresh eyecups (post mortem time less than 48hrs) formed a suspension of pigmented cells when cultured in 24-well plates. After one week incubation at 37°C (95% air + 5% CO2), some of cells had begun to attach to the bottom of the well. This was confirmed using an inverted microscope (Figure 2.1). Figure 2.1 showed that the cells had very clear cytoplasm and typical RPE irregular shape, there were some yellow brown pigments could still be seen, but the pigment disappeared after a few passage cultures.

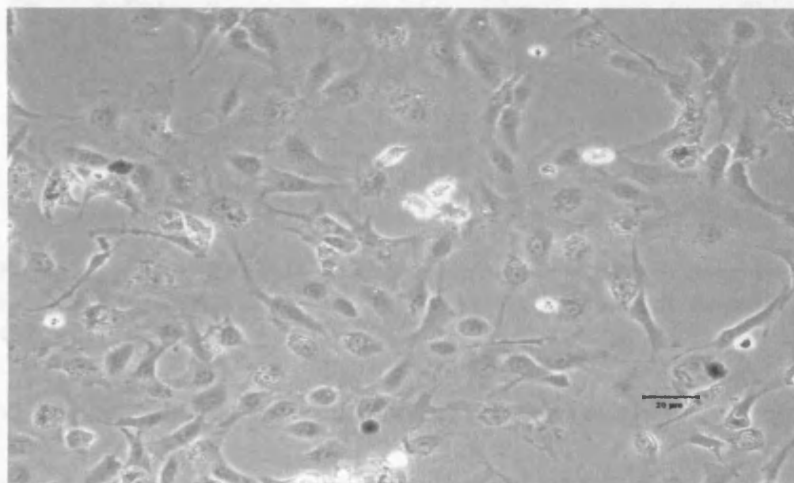


Figure 2.1 Primary RPE cells isolated from a 58 year's old male donor cultured on 24-well plate after 12 days. Magnification x20

Two weeks later, more RPE cells had attached and the previous attached cells had begun to divide and form new colonies of primary RPE cells. There was no fibroblast cells contamination. If contamination occurred with choroidal fibroblastes, these could be identified under the microscope due to their

formation of spindle-shaped cells with a striated appearance (cultures would be discarded).

By the third week, the RPE cells had divided to become nearly confluent in the wells (figure 2.2). At that point, they were harvested by following standard protocol as described previously, and sub-cultured in T75cm^2 flasks containing 10% FCS + 1% antibiotics + 1 % fongizone in Ham F10 growth medium.

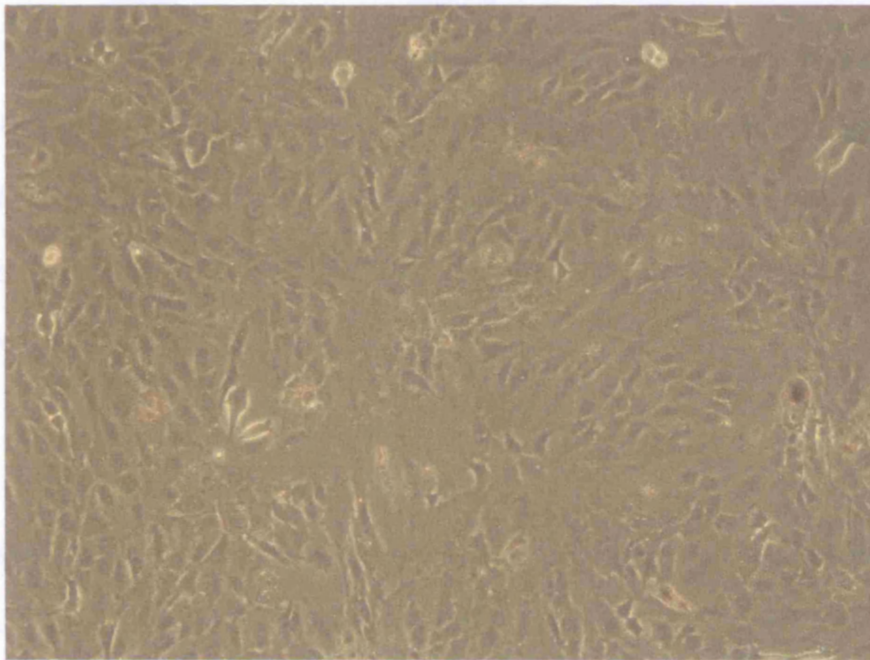


Figure 2.2 Primary RPE cells isolated from a 58 year's old male donor cultured on 24-well plate after 21 day and cells were nearly confluent. Magnification x20

When the cells reached confluence in the flasks, they were harvested as previously described and used for experimental purposes or stored in liquid nitrogen.

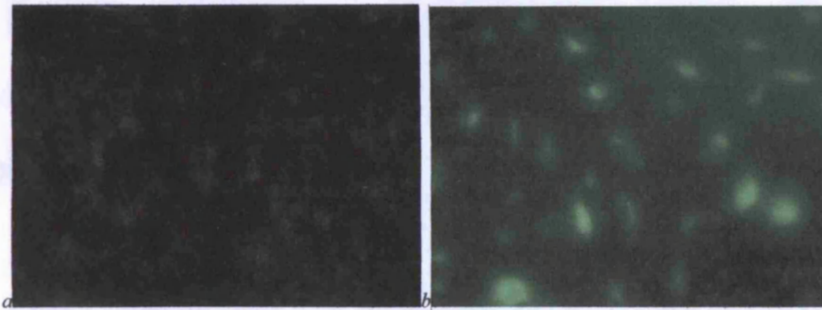


Figure 2.3 Negative (a) and positive (b) staining for pancytokeratin in primary RPE cell culture. Magnification x40

Upon immunostaining of RPE cells, the cultures that stained positive for pancytokeratin fluoresced green under UV light using the TRITC filter in figure 2.3 b. This confirmed the purity of primary RPE cell cultures. Negative controls in figure 2.3(a) did not stain for pancytokeratin and thus there was no fluorescence observed.

2.4.1.2 ARPE-19 Cell culture

ARPE-19 cells have similar morphologies to primary RPE cell culture (figure 2.4). They reached confluence in 6-well plates in just 3-4 days and in T75cm^2 flasks in approximately 4-6 days in 10% FCS containing growth medium (figure 2.5).

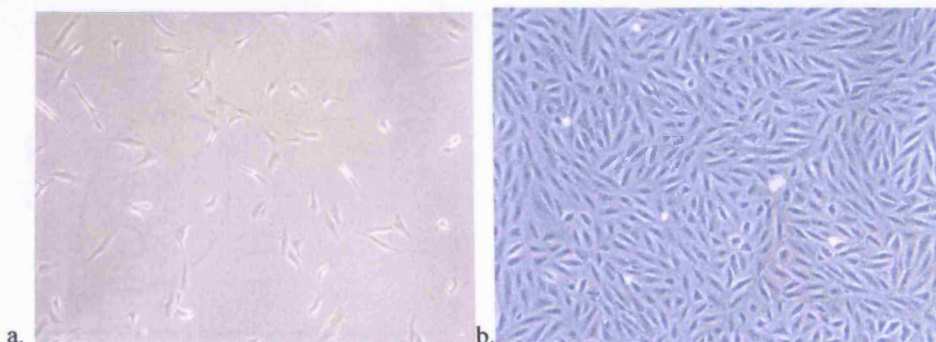


Figure 2.4: ARPE-19 was cultured after 2 days from splitting (a) and cultured after 7 days splitting (b). Magnification x20

After reaching confluence, they cells were trypsinised and either stored in liquid nitrogen as described for primary RPE cells or maintained at confluence in 6-well plates for experimental purposes.

2.4.2 Flow cytometry for Analysis of Auto fluorescent Granules Generated in Cultured RPE cells (Primary and ARPE-19) Following Challenge with POS

2.4.2.1 Flow Cytometry

The accumulation of auto-fluorescence granules was examined by flow cytometry. The excitation wave length was 488nm and emission wavelength was 530nm. In order to find out what would be the ideal concentration to feed the RPE by POS to mimic the pathophysiological conditions *in vivo,* RPE cells were fed by 1x 10^4/ml, 1x10^5/ml, 1x10^6/ml, 1x10^7/ml and 1x10^8/ml of POS respectively. Dot plot graph (a) showed the major similar cell populations within the gate and histograph (b) showed the increase of autofluorescent granules within the RPE cells (peak shifted from left to right).

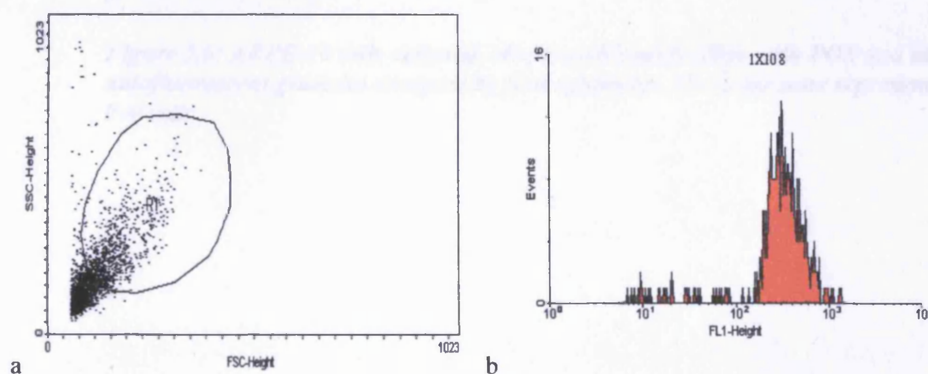


Figure 2.5: ARPE-19 cells cultured 24hours fed with 1x10^8/ml POS and auto fluorescence granules analysed by flow cytometry. The circle region represents live cell; most ARPE cells were dead after 24 hours fed with 1x10^8/ml of POS

Figure 2.5 showed that after 24 hours of the ARPE-19 cell fed with POS $1x10^8$/ml, most of the cells were dead. This could be seen in the gated region that represented the live cells. Cell death was also confirmed by staining the cells with trypan blue and checked under light microscope (dead cells showed positive blue staining). By comparing with cells fed with $1x10^6$/ml and $1x10^7$/ml POS and control, there was a significant increase of autofluorescence granules generated in ARPE-19 cells (median=279.1) even though there were so many cells dead. This suggested that the $1x10^8$/ml concentration POS were too high to mimic the *in vivo* environment of RPE and may not be suitable for use in the following experiments. ARPE-19 cells fed with $1x10^6$/ml and $1x10^7$/ml POS showed that cells could survive well even cultured until 28 days. Flow cytometry results are shown in figures 2.6- 2.17.

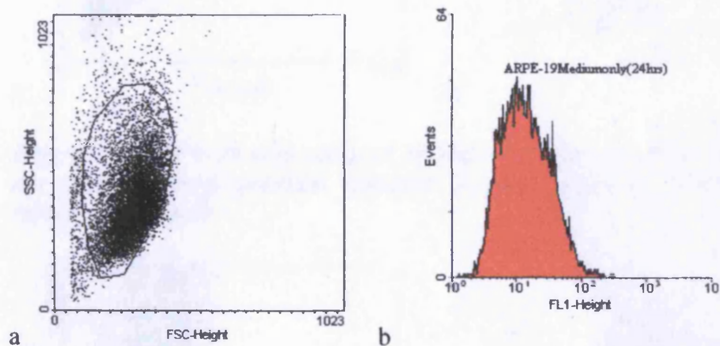


Figure 2.6: ARPE-19 cells cultured 24hours without feeding with POS and the autofluorescent granules analysed by flow cytometer. Circlular zone represents live cells.

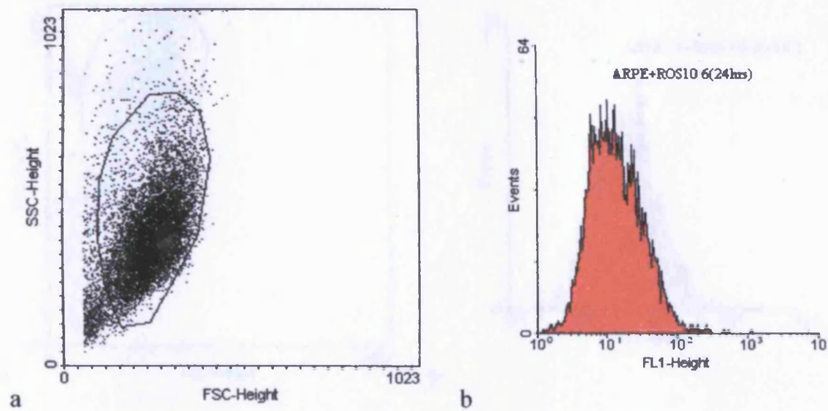


Figure 2.7: ARPE-19 cells cultured 24 hours feeding with POS 1x10^6/ml and the autofluorescent granules analysed by flow cytometer. Circular zone represents live cells.

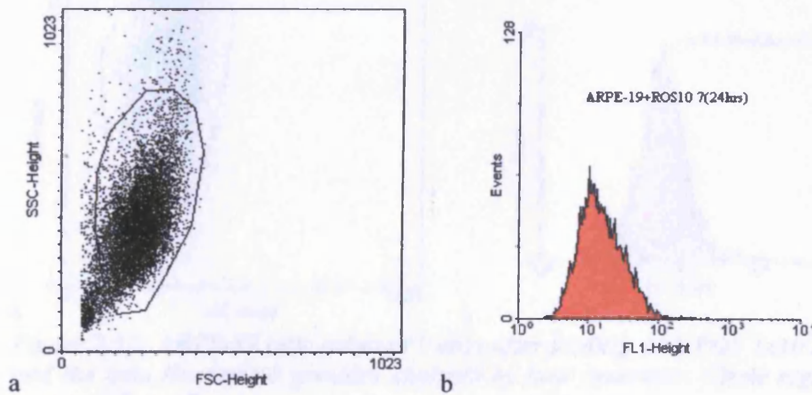


Figure 2.8: ARPE-19 cells cultured 24 hours feeding with POS 1x10^7/ml and the autofluorescent granules analysed by flow cytometer. Circular region represents live cells.

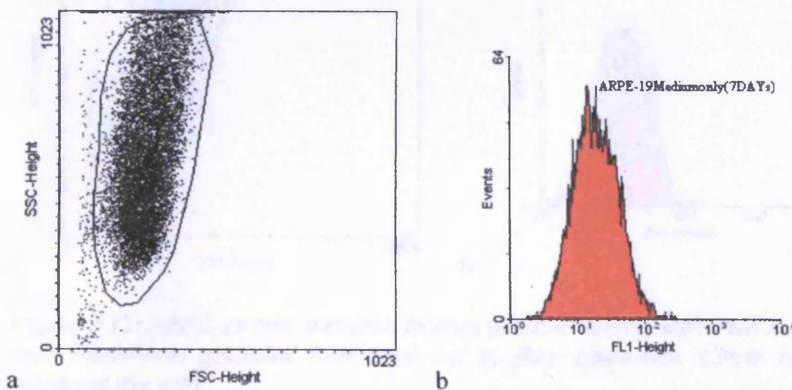


Figure 2.9: ARPE-19 cells cultured 7 days without feeding with POS and the autofluorescent granules analysed by flow cytometer. Circle region represent live cells.

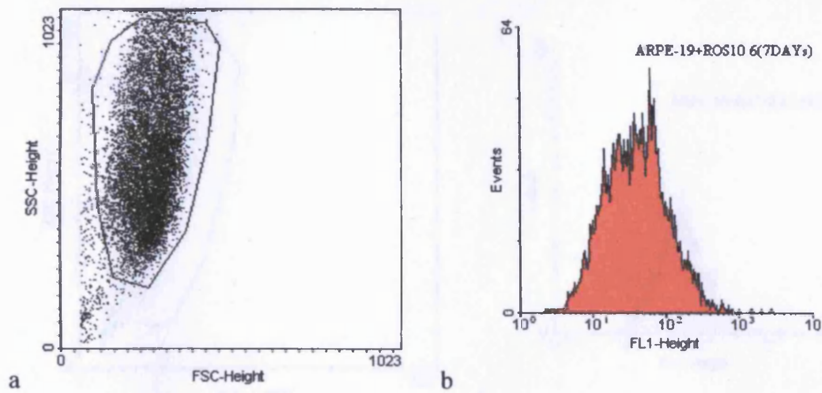


Figure 2.10: ARPE-19 cells cultured 7 days after feeding with POS 1x10^6/ml and the autofluorescent granules analysed by flow cytometer. Circle region represent live cells.

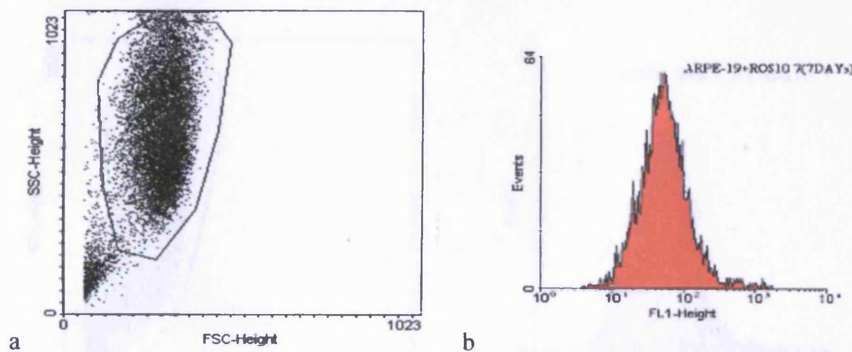


Figure 2.11: ARPE-19 cells cultured 7 days after feeding with POS 1x10^7/ml and the auto fluorescent granules analysed by flow cytometer. Circle region represent live cells.

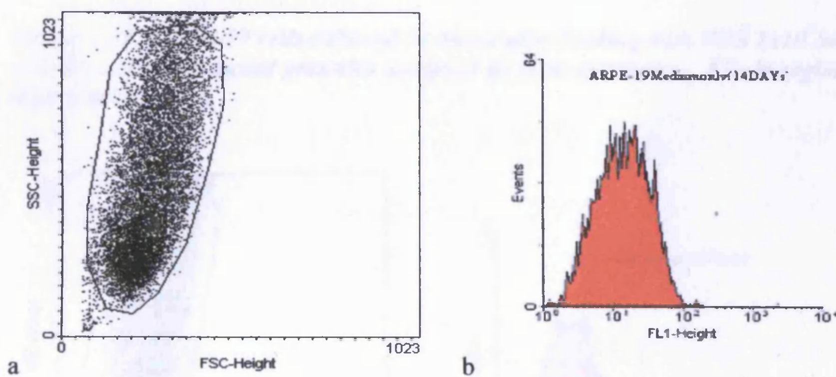


Figure 2.12: ARPE-19 cells cultured 14 days without feeding with POS and the auto fluorescent granules were analysed by flow cytometer. Circle region represent live cells

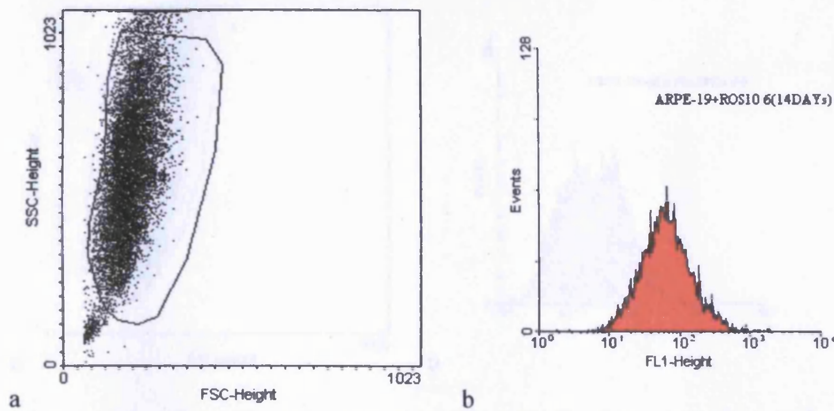


Figure 2.13: ARPE-19 cells cultured 14 days after feeding with POS $1x10^6$/ml and the autofluorescent granules analysed by flow cytometer. Circle region represent live cells.

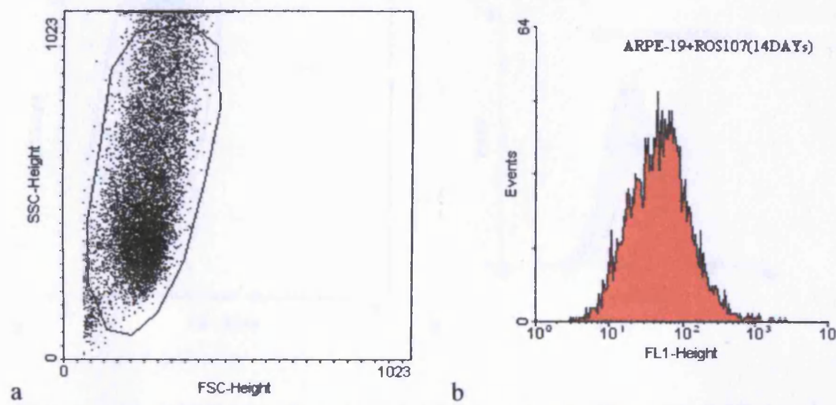


Figure 2.14: ARPE-19 cells cultured 24 hours after feeding with POS $1x10^7$/ml and the auto fluorescent granules analysed by flow cytometer. Circle region represent live cells.

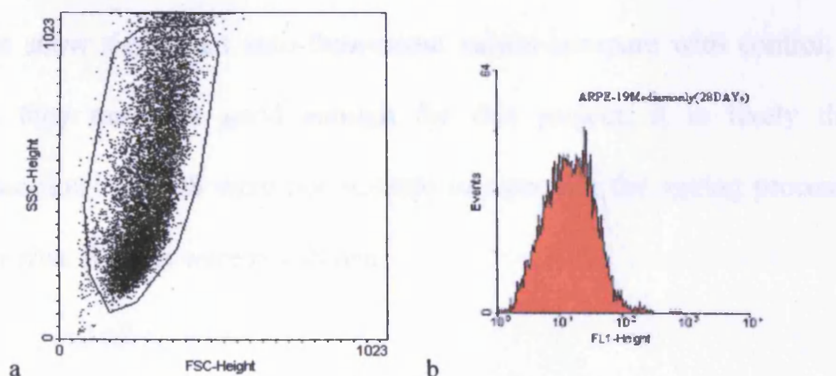


Figure 2.15: ARPE-19 cells cultured 28 days without feeding with POS and the autofluorescent granules were analysed by flow cytometer. Circle region represent live cells.

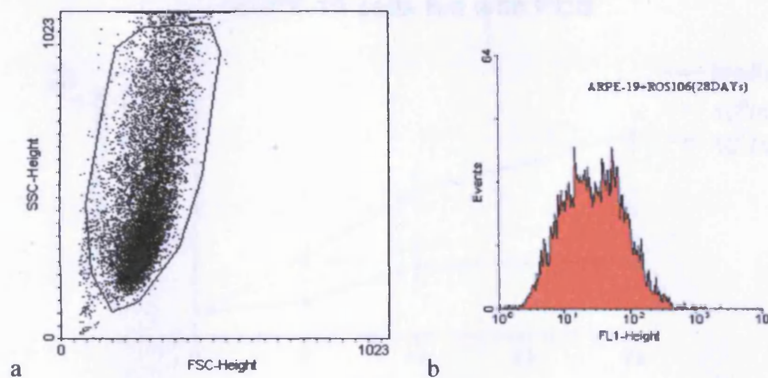


Figure 2.16: ARPE-19 cells cultured 28 days after feeding with POS 1x10⁶/ml and the auto fluorescent granules were analysed by flow cytometer. Circle region represent live cells.

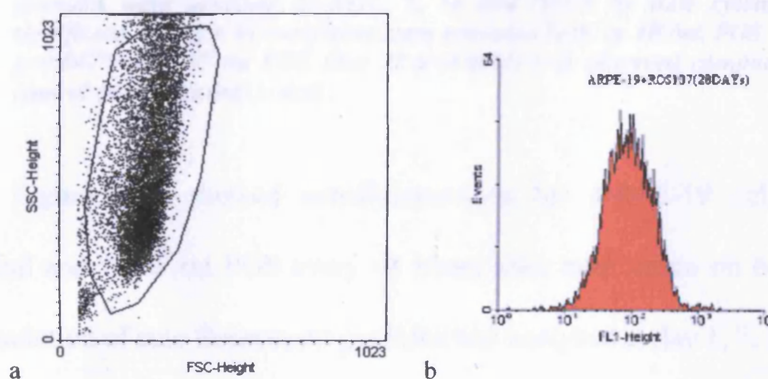


Figure 2.17: ARPE-19 cells cultured 28 days after feeding with POS 1x10⁷/ml and the auto fluorescent granules were analysed by flow cytometer. Circle zone represent live cells.

POS of $1x10^4$/ml and $1x10^5$/ml were used for the experiment but results did not show significant auto-fluorescent values compare with control, which means they may not good enough for this project. it is likely that the concentrations of POS were not suitable to represent the ageing processing in RPE *in vivo.* Results were not shown.

characteristic or age-related changes, the isolated primary RPE cells from a 58 year old male donor were also used in the same treatment conditions. Results showed a similar trend compared with that shown in figure 2.19. These results support our previous results in ARPE-19 cell line. As there were not enough cells from this donor, experiments could not be repeated from this donor cells, so there was not enough statistics available to tell if the increase was significant or not.

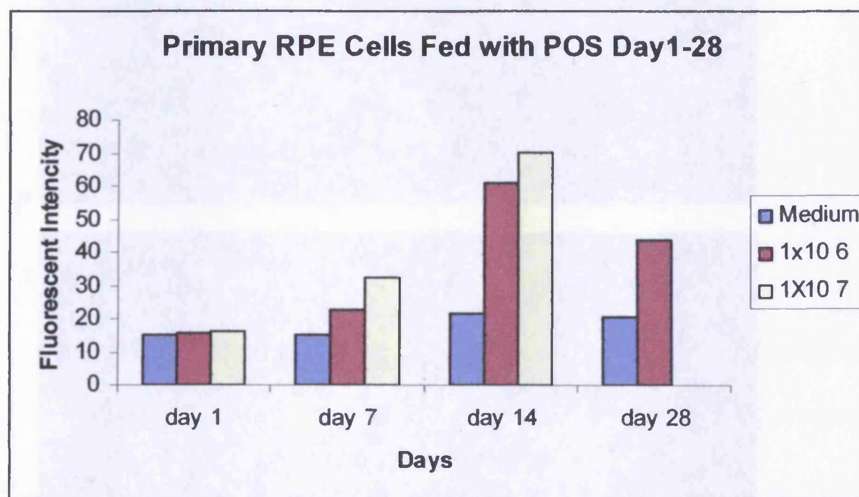


Figure 2.19: Primary RPE cells (58 year-old male donor) fed with $1x10^6$/ml and $1x10^7$/ml POS every 48 hours after confluence for 1, 7, 14 and 28 days. Auto fluorescent granule accumulation were analysed by flow cytometry. Cells were dead after 14 days of challenge by $1x10^7$/ml (no statistic data).

It is unknown why after 14 days feeding with 10^7/ml POS cell death occurred. Repeat experiments are necessary to determine this by using the other donor's cells. Results showed in section 2.4.3.1.

2.4.2.2 Light/Fluorescence microscopy and photography

ARPE-19 and primary RPE cells fed with POS $1x10^6$/ml and $1x10^7$/ml after day 1, 7, 14 and 28 were also checked under an invert light/fluorescence microscope. Under the light microscope, after 1 day culture the cells look growing well with clear cytoplasm, similar with control. After day 7, 14 and 28,

from the figure 2.20, there was a high density of dark granules (size varies) accumulation seen in the RPE cell cytoplasm and the increase was POS dose dependent. Some granules were also observed in the control group but not as much as cells fed with POS.

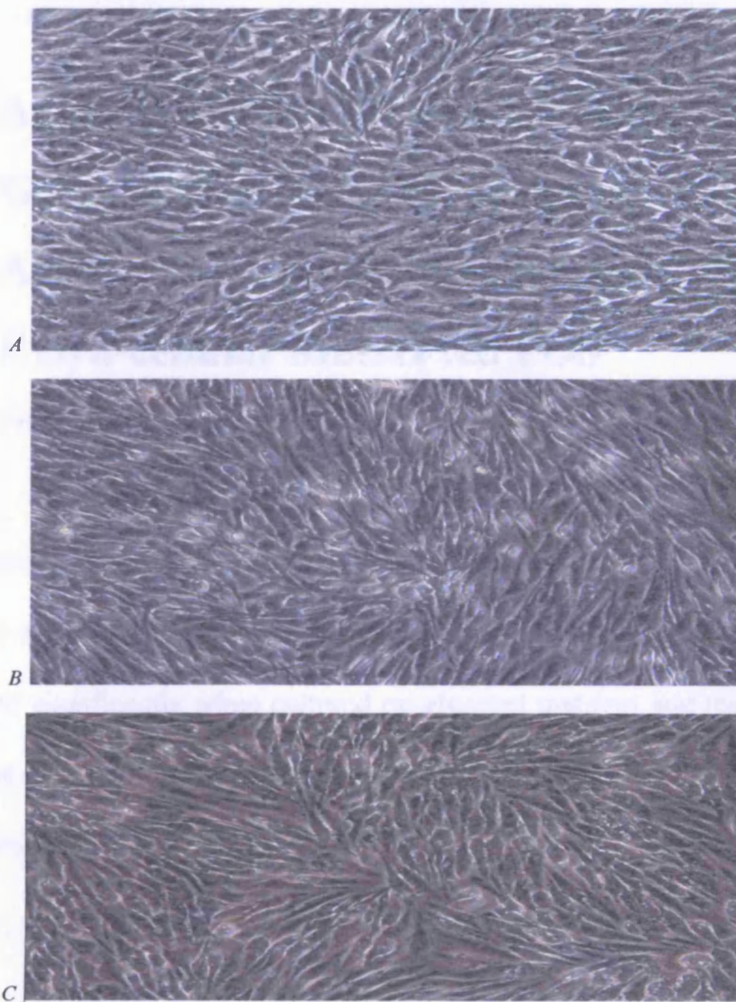


Figure 2.20: ARPE-19 cells after feeding with POS for 7 days. Cells were washed with PBS and photographs were taken under an invert light/fluorescent microscope. (a): cells withoutt fed with POS (b) Cells fed with 10^6/ml POS and (c) cells fed with 10^7/ml POS. Magnification x40

Photographs in figure 2.20 a, b and c show the cells after 7 days feeding with and without POS (10^6/ml and 10^7/ml). The photographs could not be taken

under fluorescence microscope as there were only yellow-green background colour could be seen. This might because the auto-fluorescent granules were not large enough to be seen under microscope or the magnification of the microscope was not big enough.

2.4.3 Analysis of Autofluorescent Granules Generated in RPE Cell (Primary and ARPE-19) Cultured on Advanced Glycated Extra-cellular Matrix fed POS

2.4.3.1 Flow cytometry

Based on the pilot study's protocol, the ARPE-19 cell lines and primary cells were treated as section 2.4.2. Similar results were seen for cells cultured on glycated matrigel after 28 days feeding with POS. Cell autofluorescence was increased significantly when cultured on glycated matrigel and they were POS, time and glycation concentrations dependent. See figures 2.21; 2.22; 2.23. (P values less than 0.01)

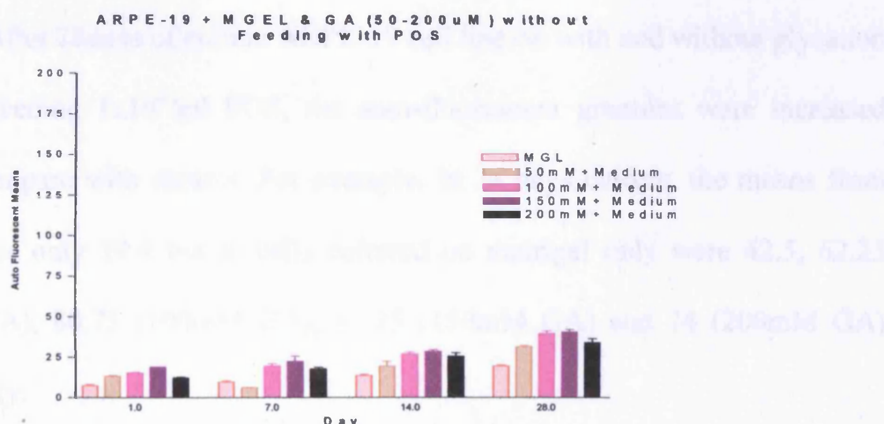


Figure 2.21: ARPE-19 cells were cultured on glycated matrigel plates for 28days without feeding POS. Accumulation of auto fluorescent granules was analysed at day1, 7, 14 and 28 by flow cytometry

After 28days of culture ARPE-19 cell line on with and without glycation matrigel only with no feeding POS, the auto-fluorescent granules were increased greatly compare with control. For example, in 28 days culture, the means from control was only 19.4 but in cells cultured on matrigel only were 30 (50mM GA), 39.5 (100mM GA), 42 (150mM GA) and 44 (200mM GA) respectively.

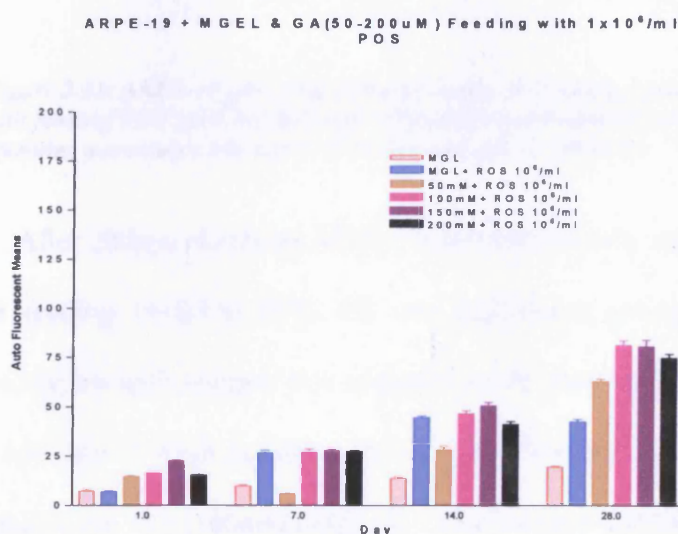


Figure 2.22: ARPE-19 cells were cultured on glycated matrigel plates for 28days with feeding POS $1x10^6$/ml every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry

After 28days of culture ARPE-19 cell line on with and without glycation matrigel feeding $1x10^6$/ml POS, the auto-fluorescent granules were increased greatly compare with control. For example, in 28 days culture, the means from control was only 19.4 but in cells cultured on matrigel only were 42.5, 62.25 (50mM GA), 80.75 (100mM GA), 80.25 (150mM GA) and 74 (200mM GA) respectively.

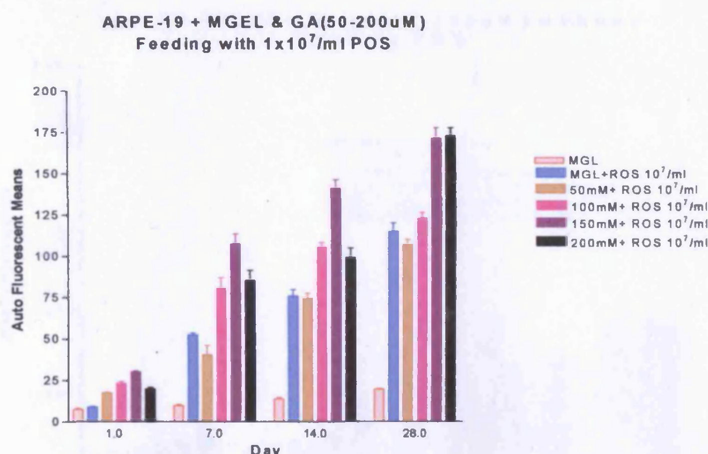


Figure 2.23: ARPE-19 cells were cultured on glycated matrigel plates for 28days with feeding POS 1x10^7/ml for every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry

After 28days of culture ARPE-19 cell line on with and without glycation matrigel feeding 1x10^7/ml POS, the auto-fluorescent granules were increased greatly compare with control. For example, in 28 days culture, the means from control was only 19.4 but in cells cultured on matrigel fed with POS were 114.25, 106 (50mM GA), 122 (100mM GA), 171 (150mM GA) and 172.25 (200mM GA) respectively. The p values are less than 0.001 compare with the day 1 culture.

As we concern that the cell line may not totally represent the true characteristics or age-related changes, also as section 2.4.2.1 unfinished work in primary cells, the other two primaries RPE cells were achieved and used in our experiments by using the same protocol. Results showed the very similar as it showed in cell line. See figures 2.24; 2.25; 2.26; 2.27; 2.28; 2.29.

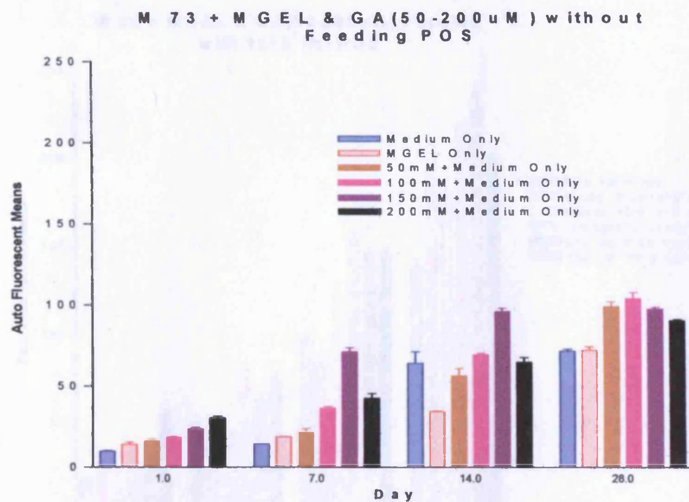


***Figure 2.24:** Primary RPE cells from a 73 year old male were cultured on glycated matrigel plates for 28days witout feeding POS. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry*

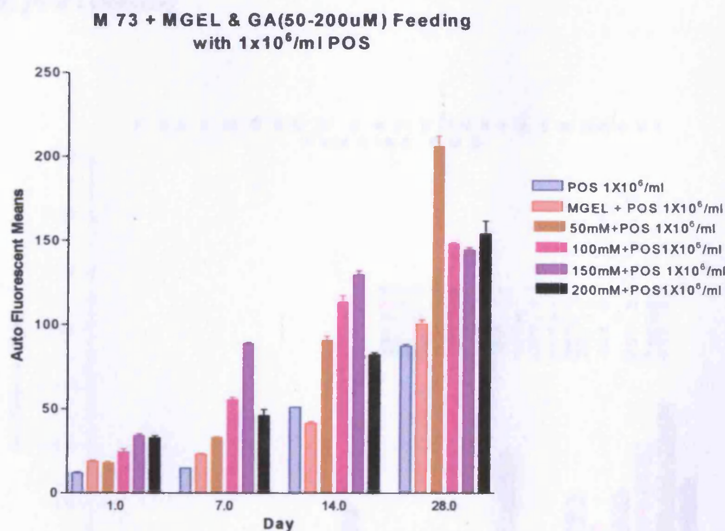


***Figure 2.25:** Primary RPE cells from a 73 year old male were cultured on glycated matrigel plates for 28days with feeding POS $1x10^6$/ml every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry*

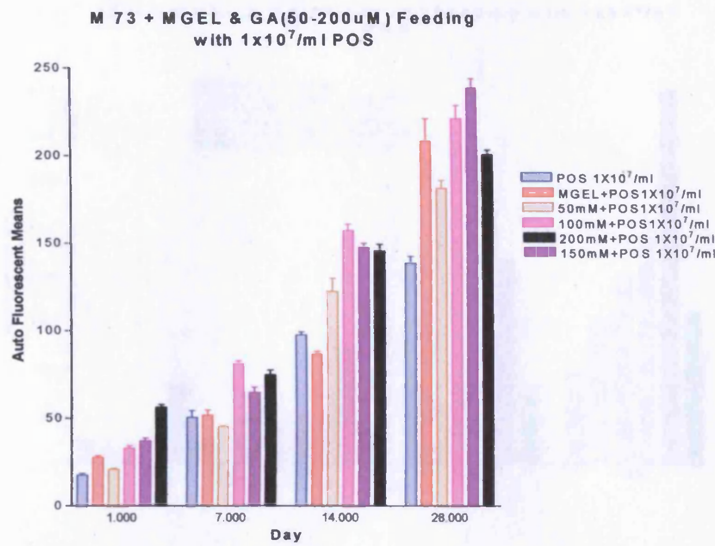


Figure 2.26: Primary RPE cells from a 73 year old male were cultured on glycated matrigel plates for 28days with feeding POS 1x10⁷/ml every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry

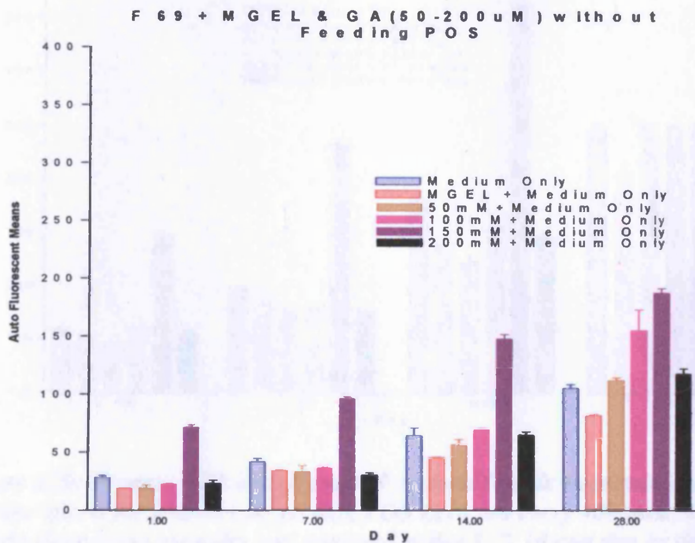


Figure 2.27: Primary RPE cells from a 69 year old female were cultured on glycated matrigel plates for 28days without feeding POS. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry

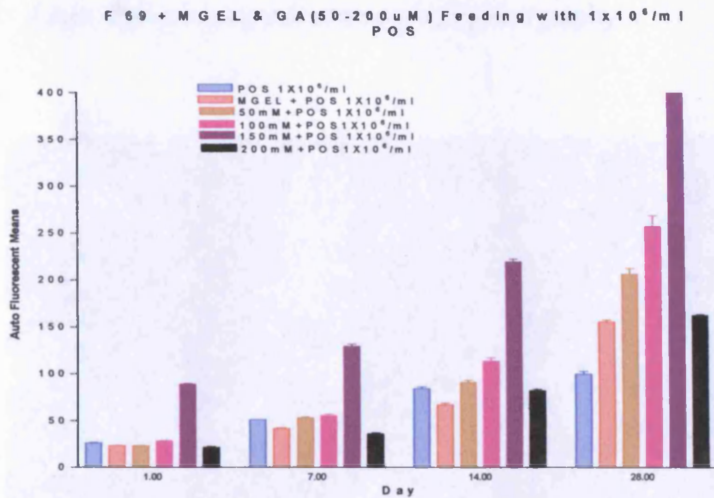


Figure 2.28: Primary RPE cells from a 69 year old female were cultured on glycated matrigel plates for 28days with feeding POS $1x10^6$/ml every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and 28 by flow cytometry

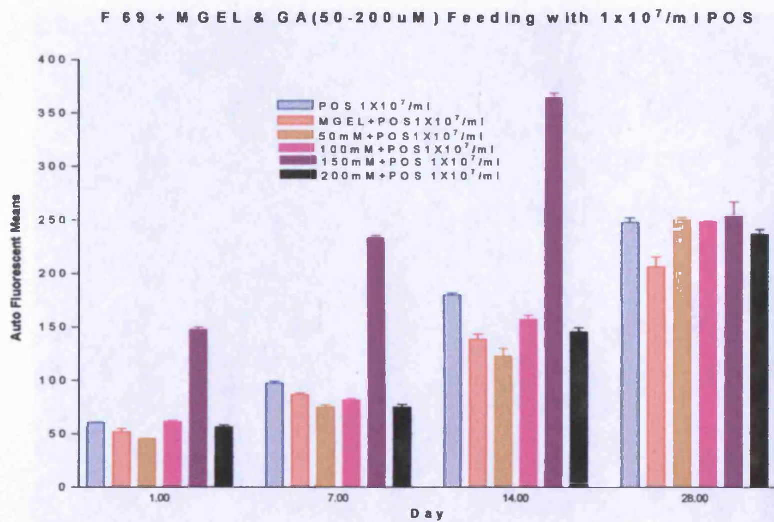


Figure 2.29: Primary RPE cells from a 69 year old female were cultured on glycated matrigel plates for 28days with feeding POS $1x10^7$/ml every 48hours. Accumulation of auto fluorescent granules was analysed at day 1, 7, 14 and day by flow cytometry

Unfortunately, at the time of the experiment, cells from young donors were not available, so we cannot address whether these changes will happen in younger people; further experiments will be done if it is possible to have cells in the future.

2.4.3.2 Light/fluorescence microscopy and photography

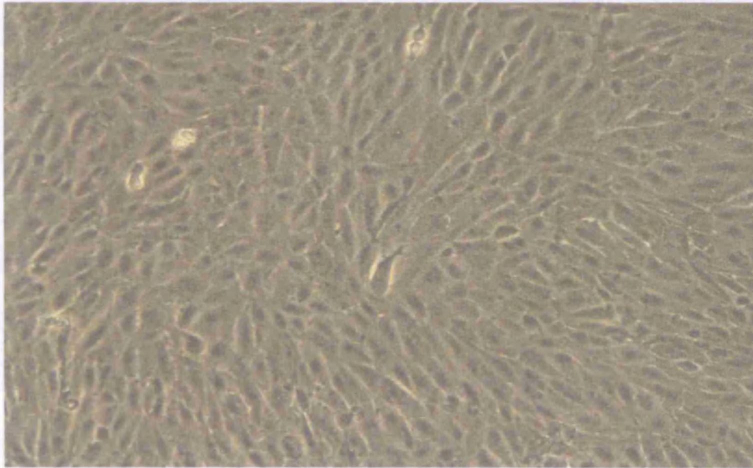


Figure 2.30: Confluent ARPE-19 Cells cultured on matrigel (without glycation) 1day without feeding with POS. Magnification x20

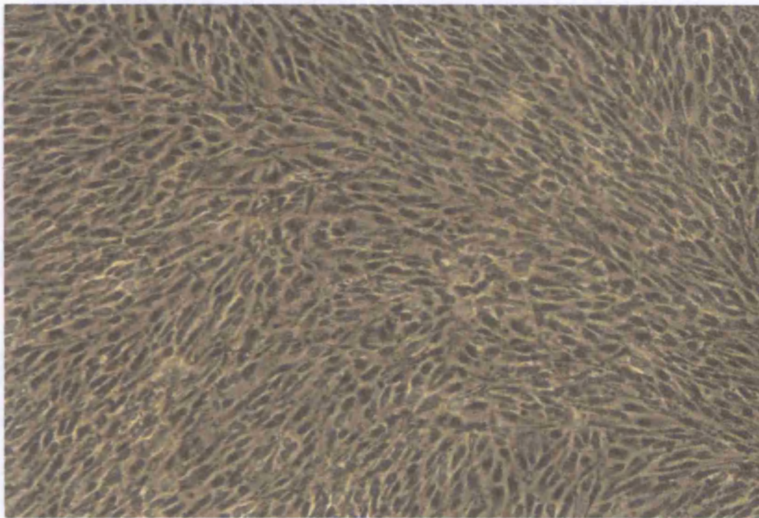


Figure 2.31: ARPE-19 Cells cultured on matrigel (without glycation) 14 days without feeding with POS. A large number of dark granules accumulated in cytoplasm in RPE and cell death happened. Magnification x20

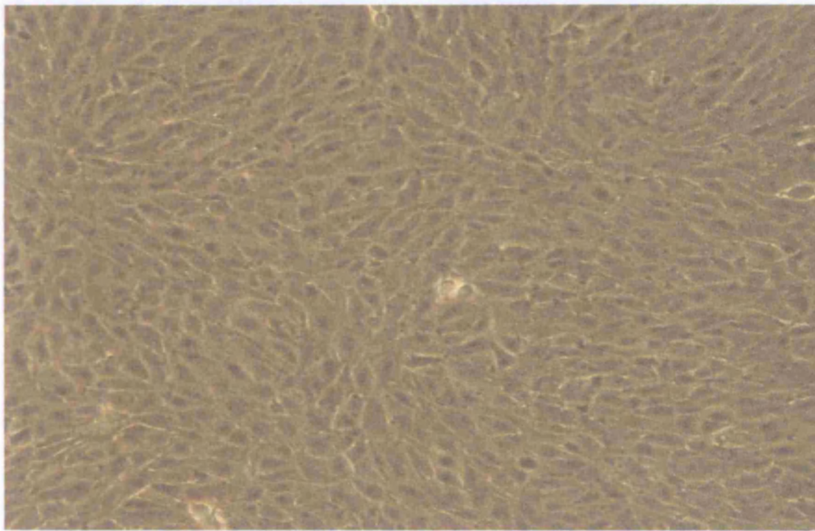


Figure 2.32: ARPE-19 Cells cultured on glycated matrigel (200uM) 1 day after feeding with $1x10^6$/ml POS. Dark granules accumulated in cytoplasm in the RPE. Magnification x20

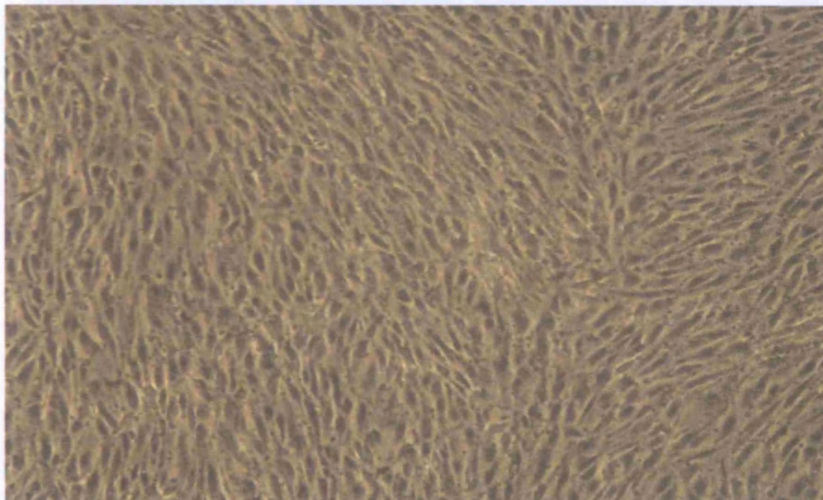


Figure 2.33: ARPE-19 Cells cultured on glycated matrigel (200uM) 14 days after feeding with $1x10^6$/ml. A large number of dark granules accumulated in cytoplasm in the RPE and cell death occurred. Magnification x20

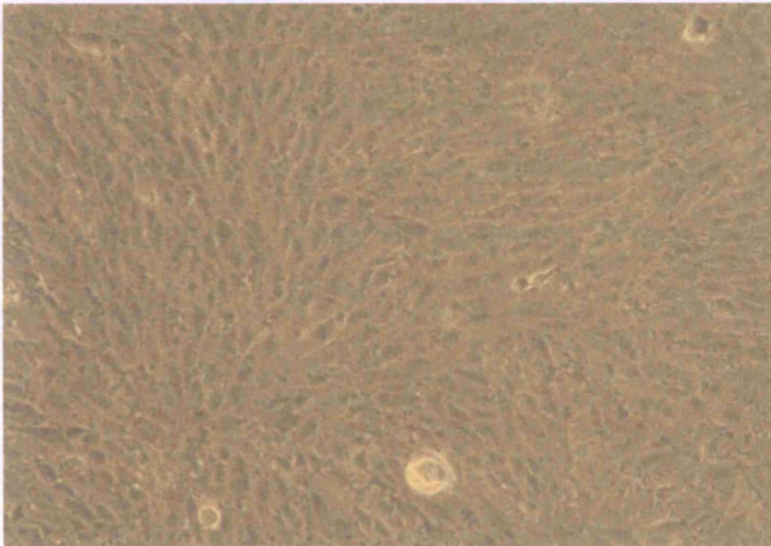


Figure 2.34: ARPE-19 Cells cultured on glycated matrigel (200uM) 1 day after feeding with 1x10^7/ml. Dark granules accumulated in cytoplasm in the RPE. Magnification x20

Figure 2.35: ARPE-19 Cells cultured on glycated matrigel (200uM) 14 days after feeding with 1x10^7/ml. A large number of dark granules accumulated in cytoplasm in the RPE and most of cells were dead. Magnification x20

Figures 2.30-2.35 The photographs of ARPE-19 cells cultured on glycated matrigel (200umM) and matrigel only (figure 3.25) with and without feeding with POS (two doses) were taken after 1 and 14 days culture. There was a clearly evidence to say that glycation could increase the granules accumulation in

cells after long periods culture. This support our method that glycated matrigel could reflect *in vivo* conditions. Unfortunately, fluorescent images still could not take it under fluorescent microscope (the same reason as before) but the flow cytometry results did demonstrate that glycation might have a key role in ageing process in human.

2.5 Discussion

To our knowledge, this study was the first time to examine the AGEs/ALEs adducts on human RPE function *in vitro*. We had investigated the effects of POS (low and high doses) and AGEs on the human retinal pigment epithelium cell function by using the AREP-19 cell line. Primary isolated human RPE cells were also cultured and used in this study to support cell line results.

There were two controls in our study; cells were not given any treatment during the culture period, and cells were cultured on "matrigel only" without the other treatments. In this study, we used light/fluorescent microscopy and flow cytometry to measure whether the AGEs accumulation in RPE with ageing could cause cell dysfunction *in vitro*, which expressed by increase auto-fluorescent in the cytoplasm to address our hypothesis.

The significant characteristic of RPE cell ageing was the accumulation of autofluorescent granules, which had been addressed it was lipofuscin that could cause RPE cells apoptosis and death. Lipofuscin accumulation in the RPE was one of the major marks of ageing in the retina. The accumulation of autofluorescent granules in cultured RPE cells had been used as a model to study lipofuscinogenesis. Using this model, experiments were designed to distinguish between these different substrates and assess their contribution to the fluorophores of autofluorescent granules generated in culture.

Our results had demonstrated this effect. Firstly, after cultured ARPE-19 cell lines 28 days with feeding POS, the auto-fluorescent granules accumulation were increased significant comparing with control. The degree was represented by

the means, for example, in the control group, the increase were only change from 8 (1 day) to 19.2 (28 days). But after the same time culture feeding with POS1x10^7/ml, the means changed from 25 (28 days) in control, to 117.25. It was time and POS dose dependent increase. It was also confirmed by using primary RPE cells.

It was hypothesised that AGEs, especially when they were immobilised on a sub-cellular matrix, could have a significant impact on RPE cell attachment and growth dynamics *in vitro*. Recent evidence suggested that advanced glycation made an important pathogenic contribution to this pathology. The AGEs accumulation and subsequent protein cross-linking occurred *in vivo* would be expected to decrease the POS indigestion, subsequently increasing lipofuscin deposit on RPE *in vivo*.

In our study, we provided the evidence to demonstrate that AGEs/ALEs had a significant role on RPE dysfunction. The results showed that after 28 days culture of these cells, especially cells cultured on glycated matrigel, the auto-fluorescent granules accumulation increased dramatically compared with cells cultured on nonglycated condition.

Overall, these data suggested that the high levels of glycated proteins present in cells from diabetics might have deleterious effects on cellular function and metabolism in such patients. These data were consistent with the report from Boulton (et al., 1998) suggesting that the essential question of whether this intervention might come how the AGEs affected the RPE function and led to RPE dysfunction with ageing.

Bruch's membrane is known to undergo considerable alteration during

ageing. It is particularly important that RPE demonstrated the most significant changes when cells were propagated on a range of AGE-modified basement membrane extract Matrigel®. Because it is known that *in vivo,* Bruch's membrane is modified by AGE, crosslinking during ageing (Handa *et al* 1999). Thus exposing RPE to an "aged" matrix mimics the *in vivo* situation.

Significantly, recent studies had demonstrated that this basal lamina became thickened (Coffey & Brownstein 1986), crosslinked and heavily AGE-modified during ageing (Handa *et al.* 1999; Farboud *et al.* 1999; Verijl *et al.* 1998) had implications with RPE dysfunction and drusen deposition. On the whole it was clear that advanced glycation of the sub-cellular matrix was the most relevant route for AGE exposure of RPE cells *in vitro*.

The increase in autofluorescence in cells cultured in medium devoid of POS was interesting in our study. Material formation of such autofluorescent granules might be derived from autophagy, necrosis/apoptosis of other cells and components of the culture medium. As they eat up the matrix if there are lysine reactions occurring with free aldehydes intermediates and e.g. lysines in the matrix they can be formed so they are called lipofuscin like fluorophores. The variation in the amount of autofluorescence accumulating in different passage numbers might reflect differences in cell 1) phagocytic ability (Miceli et al., 1994), 2) digestive capacity (Boulton et al, 1994; Vilcox, 1988).

Unfortunately, in our light/fluorescent microscope studies, we could only see the differences after 28 days culture RPE cells that there were dark granules accumulated in cell cytoplasma under the light microscope. The fluorescent images could not be taken under fluorescent microscope to support the results from

flow cytometry. It may be because the microscope was not good enough or the fluorescent granules were not large enough to be seen under those magnificent scales as there were only yellow-green background colour could be seen.

In conclusion, this chapter had shown that AGE-modification of the matrigel had an important impact on RPE behavior. This was significant because it was the most likely route by which RPE would be exposed to AGEs *in vivo*. Overall, within the context of age-related dysfunction of the RPE, it was clear that AGEs might have an important role inducing abnormal cell behaviour and disruption of normal cell function, which had obvious implications for the pathogenesis of many RPE-related disorders.

3.1 The Effect of AGEs/ALEs on Lysosomal Activity in the RPE

3.1.1 Introduction

Lysosomal acid hydrolases are responsible for the degradation of a variety of macromolecules such as proteins, lipids, complex carbohydrates, and nucleic acids (Berman, 1994) and are much more active in RPE than in many other tissues of the body (Hayasaka 1974; Wu and Aguirre, 1997). In the RPE, these enzymes are responsible for the degradation of ingested POS materials. It is very important for the RPE to maintain healthy photoreceptor-cell function and normal vision. A single RPE cell usually can phagocytosis and degrade approximately 2000 – 4000 outer segment membranous discs everyday (Bok & Young 1969). This can explain the main reason why the RPE plays a major role in many age-related diseases of the retina. As the lysosomal system is the major system in the retina to phagocytose and degrade POS, it has been suggested that the lysosomal contributes to RPE dysfunction with ageing leading to increased lipofuscin formation and, perhaps, drusen deposition in the sub-RPE space (Hayasaka 1983).

Increasing evidence has suggested that AGEs may have a role in age-related RPE dysfunction. It is known that AGEs can be endocytosed by many cell-types, and they are directed into secondary lysosomes (Stitt *et al* 2000). It has been found that AGEs accumulated in the lysosomes compartment in other non-retinal cell types. Therefore it is reasonable to hypothesise that AGEs have an important pathophysiological effect in age-related RPE dysfunction. The research should be focused on this complex lysosomal system of the RPE cells and the effects of AGEs

on the ability of the RPE lysosomal system to degrade photoreceptor outer segments.

Lysosomes can efficiently degrade macromolecules such as proteins and lipids at an acidic pH. So far, there are over 60 types of lysosomal enzymes that have been found and many of them have been identified in the RPE. The primary substrates of the lysosomes in RPE are the protein and phospholipid-rich POS, which are degraded by proteases, phospholipases and lipases. The lysosomes of the RPE are specialised for breaking down photoreceptor outer segments. Two lysosomes, Cathepsin D and acid lipase, are higher in the RPE compared with the other ocular tissues (Hayasaka *et al* 1975). Hayasaka *et al* 1975) research found that the aspartic protease Cathepsin D played a major role in degrading outer segments as the Cathepsin D digested the protein rhodopsin which was the main glycoprotein component of photoreceptor outer segments (Hayasaka *et al* 1975).

Cathepsin B is a papain-like cysteine protease, which is one of the major components of the lysosomal proteolytic system responsible for protein degradation and turnover (Wijffels, 1998; Mort & Buttle, 1997). Cathepsin B has been suggested to play a role in growth and metastasis of many types of cancer. Movement of Cathepsin B and other lysosomal Cathepsins towards the cell membrane or their secretion outside the cell may lead to degradation of the extracellular matrix.

Acid phosphatases (APS) are a family of enzymes that are widespread in nature. They can be found in human, many animal and plant species. Although they have a common functional identify, different kinds of APS are different regarding tissue composition; chromosomal origin; molecular weight; amino acid homology; sequence length; resistance to L(+) tartrate and to fluoride. Human APS are found at low concentrations in physiological condition. However, some significant changes

in its synthesis can occur in particular diseases; they are expressed either abnormally high or low enzyme activity which has been seen as part of the pathophysiological procedure. These enzyme activity changes may suggest that APS can be used as a method to investigate the pathophysiology of the relative disease related to RPE ageing dysfunction in clinic.

The process of age-related changes of RPE lysosomes enzyme activity is unknown. The first report of an age-dependent reduction in activity of lysosomes in the RPE was for α-mannisidase (Wyszynski *et al* 1989). Similar age-related decreases in the specific activities of α-mannosidase, beta- galactosidase N-acetyl-beta-glucosaminidase, and N-acetyl beta galactosaminidase have also been reported by Cingel in 1996 (Cingle *et al* 1996). Boulton *et al* (2001) reported that there was an age-dependent decrease in the activities of acid phosphatase and Cathepsin D. Verdugo *et al* (1997) found that the activities of Cathepsin D increased with age. Animal research found that ageing rats have been shown to accumulate enzymatically inactive but immunological reactive Cathepsin D (Wiederanders & Oelke 1984). It seems clear that there are some significant changes to the RPE lysosomal system with age.

With the important role to phagocytic and degradation of POS in the RPE, it could be expected that any changes in the lysosomes system would lead to its dysfunction and would cause a significant consequences for the RPE and retina. Advanced glycation has been shown to alter the function of many different proteins and to significantly alter the activity of many other enzymes. The free amino groups of proteins, such as the ε-amino groups of lysine and arginine residues are usually the important site for maintaining the protein's physiological structure and function *in vivo*. Evidence showed these were the primary targets for advanced

glycation.

Conversely, it has also been shown in some cases that if the primary substrate of an enzyme is glycated then it is less susceptible for enzymatic degradation. For instance, non-enzymatic glycation of fibrin makes it difficult to be degraded by plasmin (Brownlee *et al* 1983) and glycated fibrinogen is more resistant to degradation by trypsin (Krantz *et al* 1987). After modification by advanced glycation, Collagen is hardly digested (Brennan 1989). Similarly, AGE modified albumin had less susceptibility to be degraded by lysosomal enzymes of phagocytes; degradation was reduced by approximately 40% which caused the accumulation of the albumin in the cells (Miyata *et al* 1997).

AGEs have been shown to inhibit lysosomal enzyme activity in other cell types. For example, lung epithelial cells exposed to glyoxal suffered a loss of Cathepsin D activity (Kasper *et al* 1999). Furthermore, the activities of lysosomal Cathepsin L, B and H also showed a dose-dependent decrease after exposure to AGE-BSA in kidney proximal tubule cells (Sebekova *et al* 1998). In macrophage lysosomes, the degradation of AGE modified LDL is much slower than degradation of acetylated or oxidized LDL (Takata *et al* 1988). Therefore age-related accumulation of AGEs in the lysosomal compartment of the RPE may provide a reasonable explanation for some of the changes in the RPE that occur as a function of age.

Apart from the possibility to changes in a cells degradative capacity, it is also assumed that AGEs may affect phagocytosis of lysosomes because glycation of LDL has been shown to reduce cellular uptake by macrophages. This may be due to the modification in the e-amino group of lysine, which is important for recognition and interaction with the LDL receptor (Lorenzi *et al* 1984; Gonen *et al* 1981).

Considering the function of RPE, it is possible that any disturbance in the cell's phagocytosis function would cause a build up of outer segment-derived materials to accumulate either intracellular or within the sub retinal space.

It is a possible to hypothesis that advanced glycation end-products may play an important effect on RPE cell dysfunction, mostly through accumulating protein and phospholipids-rich POS in lysosomes and causing a deleterious influence on degradative ability and lipofuscin formation. The aims of this study were to investigate the relationship between the accumulation of AGEs in the RPE and lysosomal activity. The activity of several key lysosomal enzymes will be evaluated after cells are exposed to AGEs.

Firstly, Cathepsin D was chosen to be a candidate of lysosomes because it was widely regarded as the most important lysosomal enzyme in the RPE. However, as it was mentioned before, Cathepsin D would not be the only lysosome enzyme which would be affected by AGEs. Therefore another cysteine protease, Cathepsin B, was included in this study because previous work had shown that inhibition of cysteine protease activity could impair degradation of outer segments and led to accumulation of undigested material in the RPE (Rakoczy *et al* 1994; Katz & Shanker 1989). Additionally, acid phosphatase was also included in this study according to previous research report.

In the current study a human RPE cell-line (ARPE-19) was used as it retain the RPE cell's phagocytic capacity in culture making it a suitable model to study the effects of AGEs on phagocytosis and degradation. Also primary RPE cells from different aged donors were also used to testify the results from cell line. Briefly, Cells were exposed to the pre-formed glycation matrigel for different time periods and enzymes activities were tested by following standard protocols.

Materials &Methods

3.1.1.1 Cathepsin D assay

3.1.2.1.1 Sample Preparation

ARPE-19 Cell cultures and POS were prepared as described in section 2.2.1 and 2.2.2,

Briefly, confluent ARPE-19 cells were detached with trypsin-EDTA in 75cm^2 culture flasks and resuspened into Ham's F10 medium (10% FCS + 1% antibiotics and fungizone). After mixed well, the cells were transferred into 6-well plates which were either coated with glycated Matrigel or just plain plastic to reach confluence. During culturing time, the medium was changed every 3rd day until the cells were confluent. After confluence, the culture medium was changed to Ham's F10 medium (2% FCS + 1% antibiotics and fungi zone) for 24 hours. The Ham's F10 medium (2% FCS + 1% antibiotics and fungizone) was used continuously until the end of experiments.

POS were diluted with Ham F10 medium (2% FCS + 1% antibiotics) from stock concentration to 1x10^8/ml and 1x10^9/ml. For a negative control, only 4 ml of culture medium was added in each well (2 wells). For the 1x10^6/ml sample, 3.96ml of medium and 40μl of 1x10^8//ml were added in each well (2 wells). For the 1x10^7/ml sample, 3.96ml of medium and 40μl of 1x10^9/ml POS were added in each well (2 wells). Medium with and without POS were changed every 48 hours until the end time point of the experiments (i.e. Day 1, 7, 14 and 28).

3.1.2.1.2 Cathepsin D Enzyme Assay by using ARPE-19 Cells

Cathepsin D activity was measured using haemoglobin as substrate with modifications to the method according to the protocol described by Boulton et al., 2001. The principle of this test is to utilize the ability of CAD to digest the haemoglobin substrate into Tyrosine. Released Tyrosine was assessed at 570nm using a Dias Plate Reader (Dynatech U.S.A). The activity of the CAD was quantified with DL- Tyrosine standard curve displayed as μg/ml.

Briefly, after cell cultures reached the time point of experiment, cells were washed with PBS twice to remove the medium. Then 250μl of 0.2% Triton X-100 in 50mM PO_4 buffer, pH 7.4, was added to each well and incubated at RT for 15 minutes to lyses cells. Cells then were scraped using a cell scraper and the lysates were transferred into a 1.5ml eppendorf and centrifuged at 100g for 5minutes. The supernatant was collected for measurement.

50μl of 2% haemoglobin in 0.25M Sodium Formate Buffer, pH 3.3, as substrate was added to each of 50μl of supernatant and then incubated at 37°C for up to 60 minutes. The reaction was stopped by adding 50μl of ice-cold 3% TCA. Further centrifugation was done at 100g for 5minutes and the proteolytic products from the digested haemoglobin in the supernatant were measured using a standard bicinchoninic acid assay kit (Pierce Chemicals, Rockford, IL, USA) which is detergent-stable. 25μl of each standard (DL-Tyrosine) and the samples (The supernatant) were added into a 96-well micro plate, then 200μl of Work-reagent was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes and then cooled at room temperature. The resulting bicinchoninic acid complex was calorimetrically assessed at 570nm using a Dias Plate Reader (Dynatech U.S.A). DL-Tyrosine equivalents were used as a calibration curve. Standard curves were

run at the same time to ensure uniformity of incubation time and substrate concentration.

To check the specificity of the assay for aspartic proteinases, pepstatin (Sigma) was added in excess (1.5μg/50μl of cell lysate) half an hour before incubation with haemoglobin substrate, and the remaining enzyme activity was measured as described above.

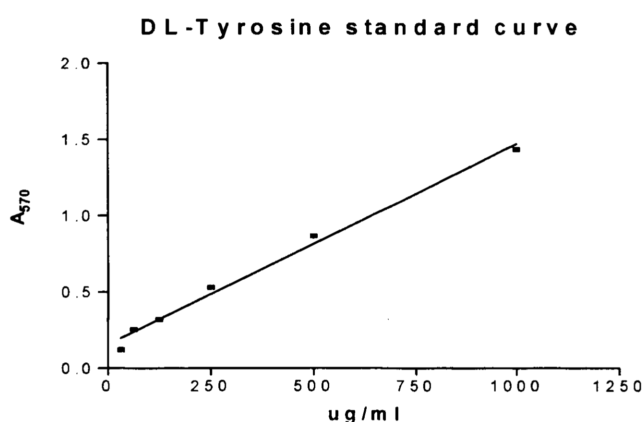


Figure 3.1 DL-tyrosine standard curve. Absorbencies were measured at 570nm.

3.1.1.2 Cathepsin B enzyme Activity Assay by using ARPE-19 Cells

3.1.1.2.1 Sample Preparation

ARPE-19 Cell cultures and POS were prepared as described in section 2.2.1 and 2.2.2,

3.1.1.2.2 Cathepsin B Enzyme Assay

This fluorometric assay has been designed for the quantitative *in vitro* determination of CAB activity in micro-plates. The test utilized the ability of CAB to digest the synthetic substrate Z-Arg-Arg (AMC). Released free AMC was determined fluorometrically at excitation wavelength 355 nm and emission wavelength 460 nm. The activity of the CAB was quantified with an AMC standard

displayed as nmol AMC/mg protein/hour

Basically, ARPE-19 cells were prepared as shown in section 2.2.1 and 2.2.2. Cathepsin B Substrate, Control Cathepsin B, Calibration Standard, Cathepsin B Inhibitor, Reduction Reagent and Assay Buffer were prepared before following the protocol. Cell Lyses Buffer, Microtitre Plate and Plate Sealer were provided by the kit.

After cell culture reached each time point of the experiment, cells were washed with ice-cold PBS twice. Then 500μl of cell lysis buffer was added in the flask and incubated on ice for 30 minutes. The lysates were collected into 1.5 ml eppendorfs and vortexed for 5 seconds. Then the lysates were centrifuged at 14,000g in a pre-cooled tabletop micro-centrifuge. After centrifugation, the supernatant was immediately transferred to a fresh microcentrifuge tube and the pellet was discarded. The lysate was diluted 1:5 before determination of the protein concentration via BCA protein assay.

All the reagents were brought to room temperature for assay. 25μl of the activation buffer was pipetted into each well (96 well plate), 50μl of standard and control Cathepsin B were added into each well (all samples are duplicated). Then the plate was pre-incubated at room temperature for 5 minutes. Then, 25μl of substrate solution was added into each well. The plate was sealed tightly and incubated at 37°C for 30 minutes. The fluorescence of free AMC was read on a fluorescence plate reader at excitation wavelength 355 nm and emission wavelength 460nm. The fluorescence measured was converted to nmol AMC using the slope of the standard curve (figure3.2)

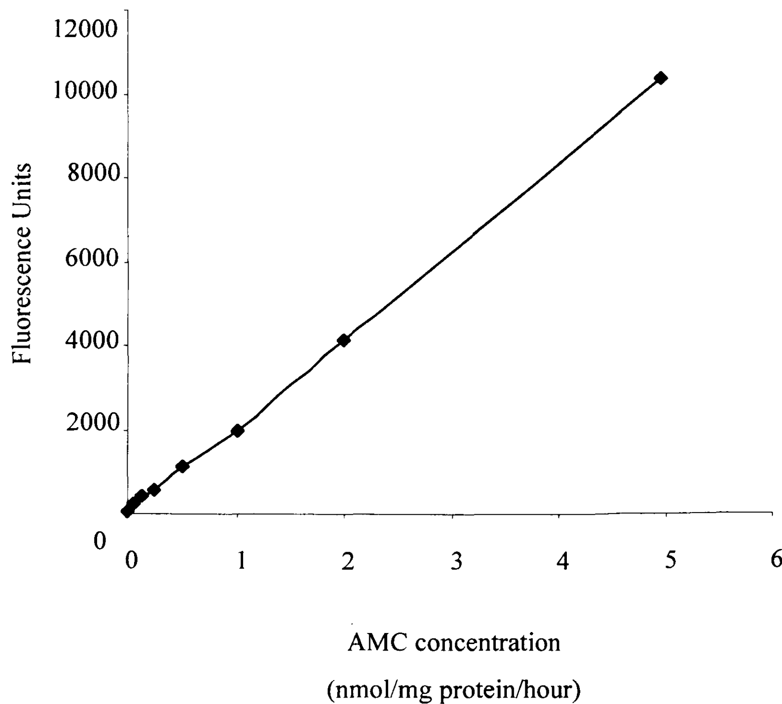


Figure 3.2 Standard curve of Cathepsin B (AMC Standard curve).

3.1.1.3 Acid Phosphotase Enzyme Activity

3.1.1.3.1 Sample Preparation

ARPE-19 Cell cultures and POS were prepared as described in section 2.2.1 and 2.2.2.

3.1.1.3.2 Enzyme Assay

Cells were lysed with 0.2% Triton X-100 in distilled water for 5 minutes. Then the lysates were incubated with 7.5mM ρ-nitrophenyl phosphate in 0.1M acetate buffer (pH 5.0) for 45 minutes at 37°C. The reaction was stopped by the addition of 100mM NaOH (pH 10.5), and the absorption of light by ρ-nitrophenol released in the reaction was measured at 405nm. By using a calibration curve for ρ-nitrophenol, the absorbance values were converted to picomoles of reaction

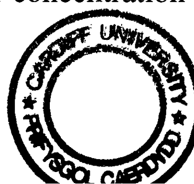
3.2 Results

3.2.1 Cathepsin D Activity

3.2.1.1 Cathepsin D activity in ARPE-19 Cell

A wide range of lysosomal enzymes are present in RPE cells, and they are responsible for the continuous digestion of phagocytosed POS *in vivo*. on the basis of enzymic studies, it has been proposed that the most important enzymes involved in the proteolysis of rhodopsin is Cathepsin D. Primary enzymic studies showing increased aspartic proteinases activity in RPE cells, which is about five times higher than that in liver, have recently been confirmed by Northern-blot analysis demonstration the up-regulation of CAD transcription (Rakocay *et al.,* 1997). It has been demonstrated by enzymic assay that inhibition of aspartic proteinases results in a significant decrease in rhodopsin proteolysis (Rakocay *et al.*, 1997). But, in spite of the CAD's significant role in the normal function of RPE cells, so far, there is very little known about the control and activation of CAD in these cells and the consequences of any changes that occur in CAD activity.

In this work, Results were shown in figure 3.4 and figure 3.5; these were mean averages from 4 independent experiments. Enzyme activities showed a dramatic increase after cells were cultured 14 days feeding with POS with and without glycation and reached its peak of activity and day 14th. From 14 to 28 days, the activities went down quickly, especially for cells challenged with POS compared with cells without challenge with POS ($p < 0.05$). For cells cultured on different glycation conditions feeding with POS, Cathepsin D activity changes showed the similar trend but had much lower extent than without glycation; they were glycation condition and POS dose dependent. There were no significant glycation concentration dependent



changes observed. Results seen from figure 3.4, there were no difference changes among GA concentrations (0-200mM). For example, after 28days culture, the enzyme activity was 0.069µg/10^5cells/min in cell cultured on 100mM GA fed with $1x10^7$/ml POS, and the control was 0.183µg/10^5cells/min in control. It was about 64% decrease)

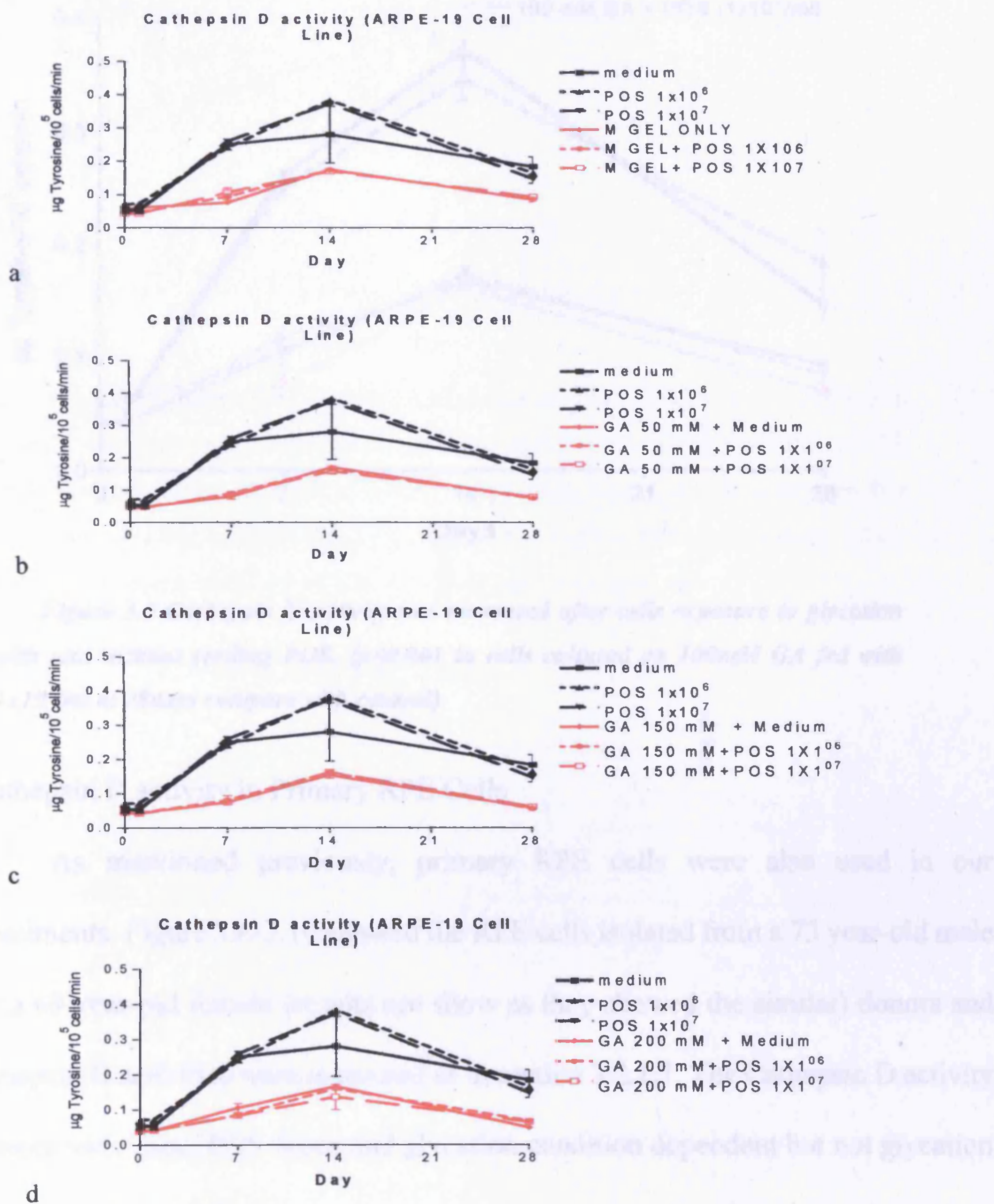


Figure 3.4 Cathepsin D activities were measured after cells exposed with and without glycation, feeding with and without POS for 28days. The changes were significant with depending on the POS dose, glycation condition and time changes. There was no glycation concentration dependent change (a: Matrigel only; b: 50mM GA; c: 150mM GA and d: 200mM GA feeding with POS [Two doses]).

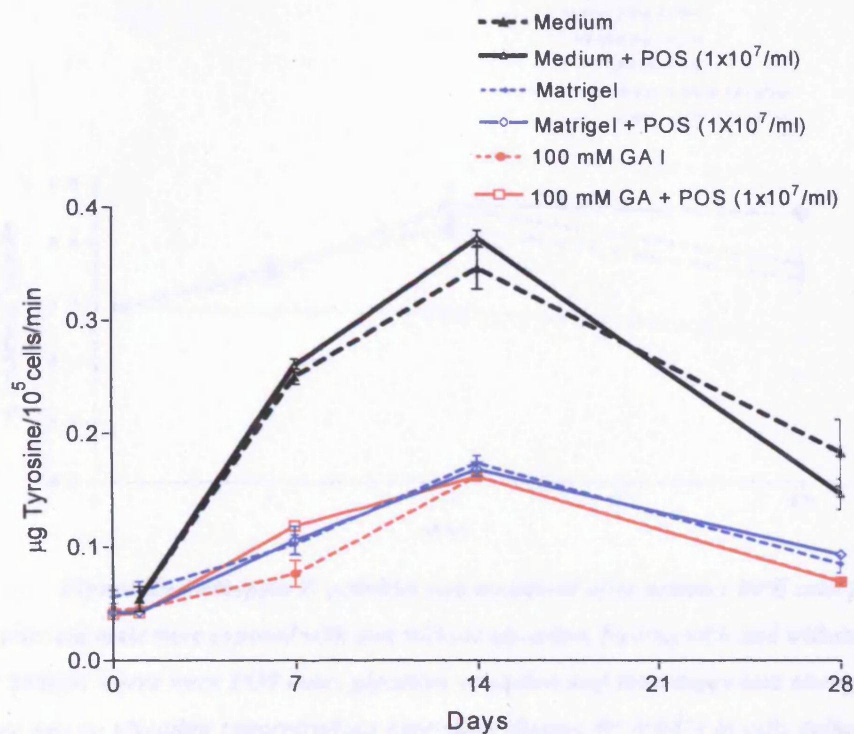


Figure 3.5 Cathepsin D activity was measured after cells exposure to glycation with and without feeding POS. ($p<0.001$ in cells cultured on 100mM GA fed with $1x10^7$/ml at 28days compare with control)

3.2.1.2 Cathepsin D activity in Primary RPE Cells

As mentioned previously, primary RPE cells were also used in our experiments. Figure 3.6-3.10 showed the RPE cells isolated from a 73 year-old male and a 69 year-old female (results not show as they showed the similar) donors and Cathepsin D activities were measured as in section 3.2.1.1. The Cathepsin D activity changes were time, POS doses and glycation condition dependent but not glycation concentration dependent.

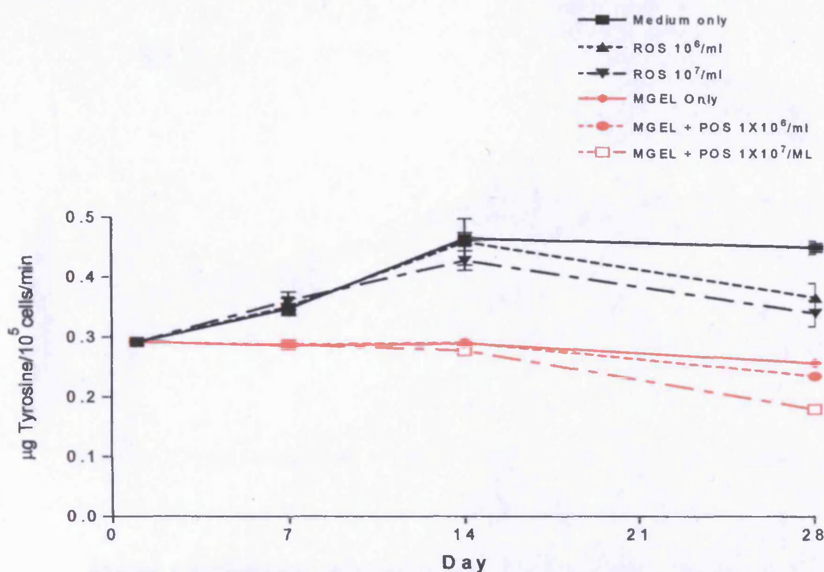


Figure 3.6 Cathepsin D activities was measured after primary RPE cells from a 73 year-old male were exposed with and without glycation, feeding with and without POS for 28days. There were POS dose, glycation condition and time dependent changes but there was no glycation concentrations dependent change. ($P=0.0014$ in cells cultured on matrigel only fed with $1x10^7$/ml POS at 28days compare with control)

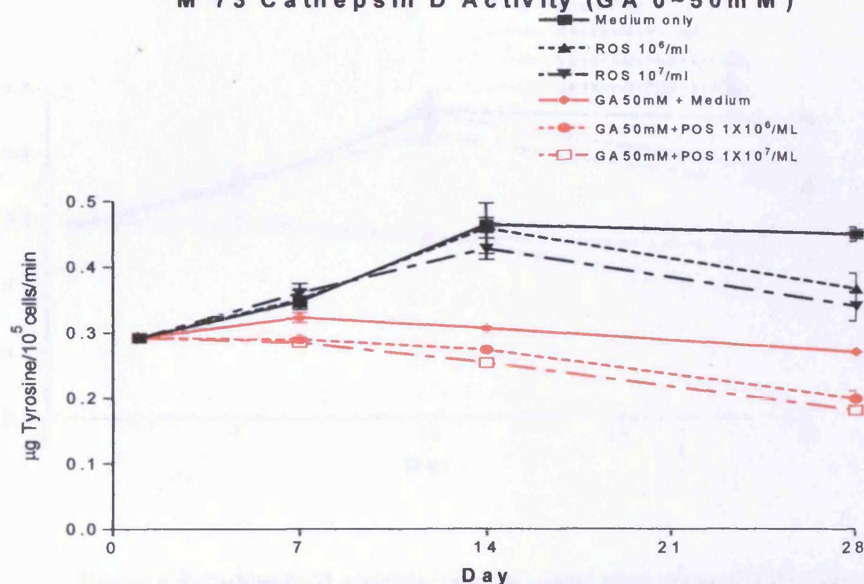


Figure 3.7 Cathepsin D activities was measured after primary RPE cells from a 73 year-old male were exposed with and without glycation, feeding with and without POS for 28days.($p=0.0017$ in cells cultured on 50mM GA fed with $1x10^7$/ml POS at 28days compare with control)

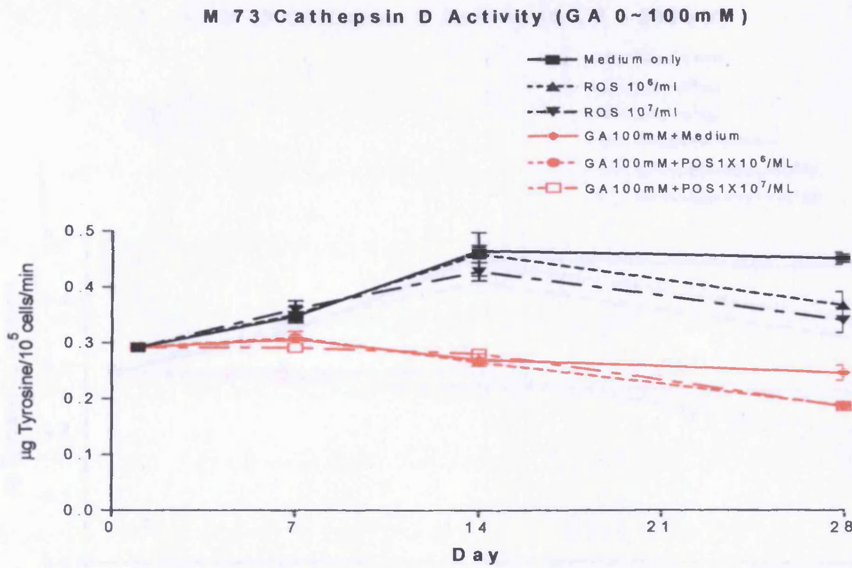


Figure 3.8 Cathepsin D activities was measured after primary RPE cells from a 73 year-old male were exposed with and without glycation, feeding with and without POS for 28days.($p<0.001$ in cells cultured on 100mM GA fed with $1x10^7$/ml at 28days compare with control)

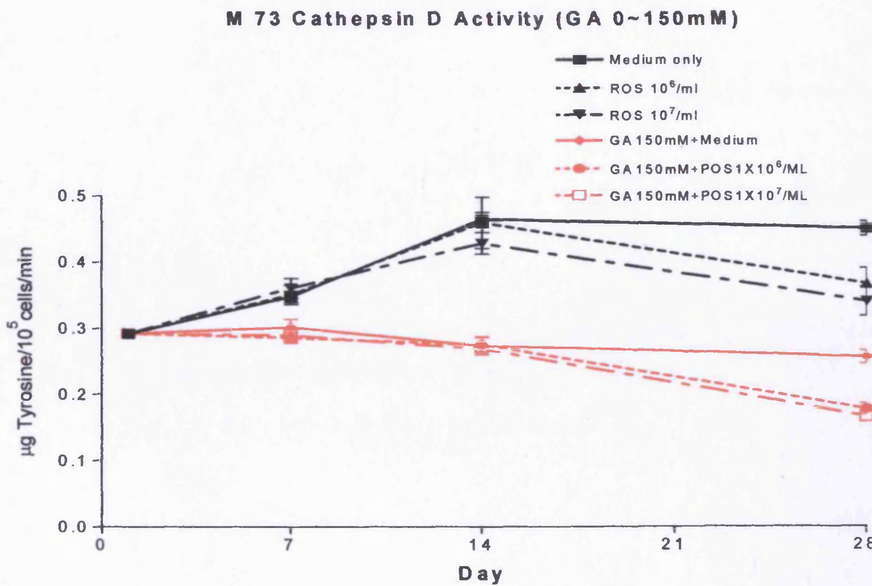


Figure 3.9 Cathepsin D activities was measured after primary RPE cells from a 73 year-old male were exposed with and without glycation, feeding with and without POS for 28days. ($p<0.001$ in cells cultured on 150mM GA fed with $1x10^7$/ml at 28days compare with control)

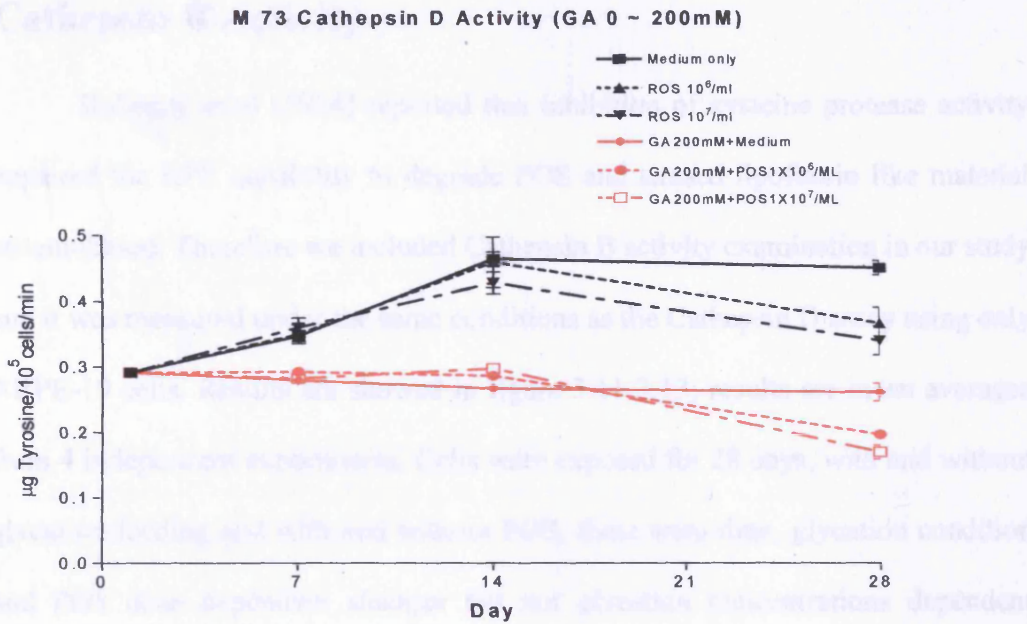


Figure 3.10 Cathepsin D activities was measured after primary RPE cells from a 73 year-old male were exposed, with and without glycation, feeding with and without POS for 28days. (p=0.0019 in cells cultured on 200mM GA fed with 1x10^7/ml at 28days compare with control)

3.2.2 Cathepsin B Activity

Rakoczy et al (1994) reported that inhibition of cysteine protease activity impaired the RPE capability to degrade POS and caused lipofuscin like material accumulation. Therefore we included Cathepsin B activity examination in our study and it was measured under the same conditions as the Cathepsin D assay using only ARPE-19 cells. Results are showed in figure 3.11-3.13; results are mean averages from 4 independent experiments. Cells were exposed for 28 days, with and without glycation feeding and with and without POS; there were time, glycation condition and POS dose dependant changes but not glycation concentrations dependent changes. ($p=0.023$ in matrigel only fed with $1x10^7$/ml POS compare with control; $p<0.001$ in 50mM GA fed with $1x10^7$/ml POS compare with control)

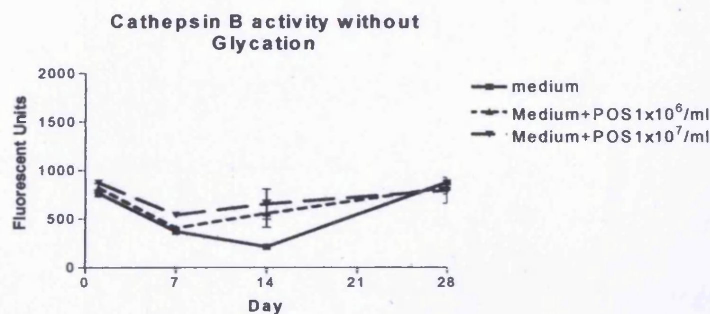


Figure 3.11 Effects of AGEs on Cathepsin B activity. Cathepsin B activity was measured after cells fed with and without POS for 28days.

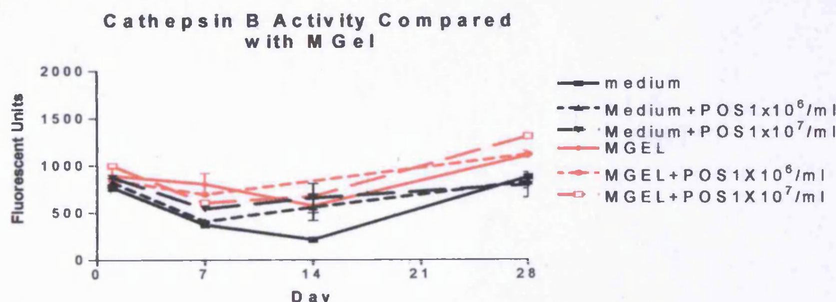


Figure 3.12 Effects of AGEs on Cathepsin B activity. Cathepsin B activity was measured after cells exposed to matrigel fed with and without POS for 28days.(($p=0.023$ in matrigel only fed with $1x10^7$/ml POS compare with control)

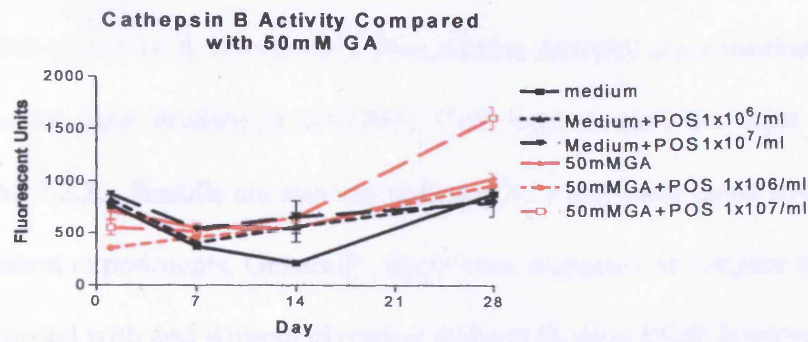


Figure 3.13 Effects of AGEs on Cathepsin B activity. Cathepsin B activity was measured after cells exposed to glycation fed with and without POS for 28days. ($p<0.001$ in cells cultured on 50mM GA fed with $1x10^7$/ml at 28days compare with control)

3.2.3 Acid Phosphatase Activity

The effects of AGEs on Acid Phosphatase Activity were measured following the protocol from Boulton et al (2001). Cells were treated the same as in section 2.2.1 and 2.2.2. Results are showed in figure 3.14 and were mean averages from 4 independent experiments. Generally, there were increases of enzyme activities after cells exposed with and without glycation without feeding POS; however, when cells were exposed to glycation and fed 28 days with POS, there was a significant decrease ($p<0.05$) of enzyme activity observed and they were time, GA condition and POS dose dependent. There were no significant glycation concentrations dependent changes observed in our experiments (results are not shown)

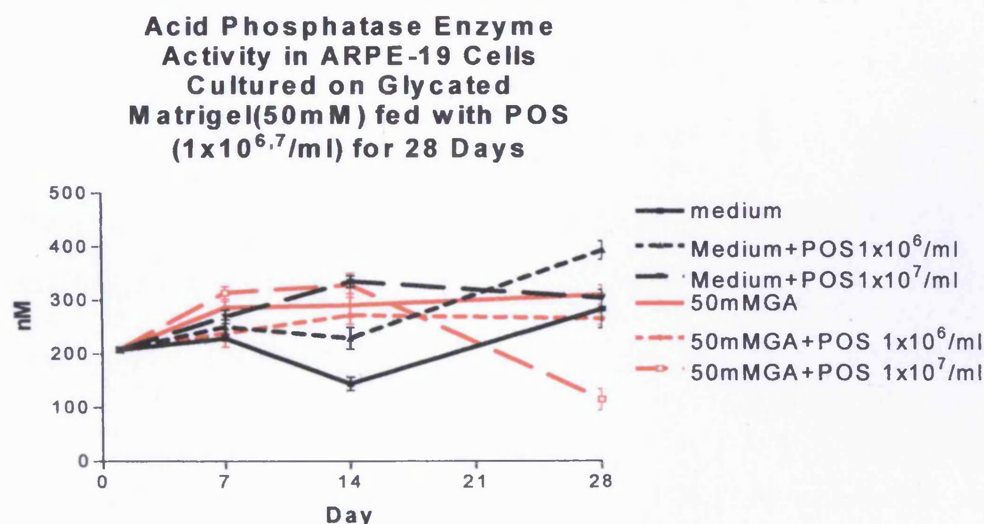


Figure 3.14 Effects of AGEs on Acid Phosphatase Activity

3.2.4 statistical Analysis

Results are expressed as mean and standard deviation which were calculated using Prism software. Statistical significance between treatments and controls were

evaluated by the one-way ANOVA (and nonparametric) unpaired t Test. A p value of less than 0.05 was considered significant.

3.3 Discussion

To what extent AGEs contribute to changes of lysosomal enzyme activity in age RPE is unclear. Many conflicting reports have shown there was both an age-related increase and decrease of lysosomal enzyme activity *in vitro* with data showing differences between species, the type of tissue analyzed and the methodology. In general; studies indicate an increase in lysosomal enzyme activity with age in the RPE. (Stitt *et al.*, 1999) This apparent contradiction of our data can be explained by the steady accumulation of lipofuscin granules with age.

Accumulation of Lipofuscin in RPE cells is the most significant change in the aging process. Due to the RPE having a very high degradative load everyday, even just a small change in enzyme activity would have severe consequences for the RPE and photoreceptors. It was hypothesised that the deposition of AGEs in RPE cells could have deleterious effects on the specialised lysosomal enzymes system degradation capability. It we could prove this, it would have obvious implications to explain the formation of lipofuscin in aged RPE. Evidence has shown that accumulation of AGEs in the lysosomes could reduce the RPE capacity to degrade photoreceptor outer segments, resulting in the accelerated accumulation of lipofuscin granules in the RPE cytoplasm.

Kasper et al (*1996, 1999*) reported that AGEs accumulation in the lung epithelial cells caused damage to the normal cell functions and renal tubular epithelium cell function as well (Gugliucci & Bendayan 1996). However, in Rakocyz's (et al 1999) report that there was no impaired Cathepsin D expression by AGE treatment and also there was no down-regulated Cathepsin D in the aged patients with AMD. In our study it has been shown that AGEs had a remarkable

effect on Cathepsin D activity. This is significant because the primary function of the RPE is to phagocytose and degrade ROS, and Cathepsin D has been widely considered as the most important protease in the RPE (Regan *et al.* 1980). Furthermore, this enzyme has already been implicated in age-related dysfunction (Boulton *et al* 1994).

Kasper *et al* have also recently demonstrated that AGEs could mediate dysfunction of Cathepsin D activity in lung epithelium (Kasper *et al* 1999) and Sebkova demonstrated that AGEs could impair protein degradation in kidney epithelial cells (Sebkova *et al* 1998). However, the association is not fully elucidated between abnormalities in RPE lysosomal function and AMD, even though it could be speculated that RPE cell's function could be impaired by the degratative enzymes which would lead to degeneration of the overlying POS. Due to the heavily load of work on the RPE and the fact that these cells must carry out this degradation job for a lifetime, even just a small changes of the enzyme activity in the cells would have a severe consequences for the RPE and photoreceptors.

This enzyme has already been implicated in age-related dysfunction (Boulton *et al* 1994; Verdugo *et al* 1997). In our study it is also shown that AGEs have a remarkable effect on decreasing Cathepsin D activity and it is significant. There was a significant increase in Cathepsin B activities in our results and it is contradictory to Sebekova's et al report in 1998; they found Cathepsin B had a AGEs dose-dependent reduced proteolytic activity after exposure of kidney epithelial cells to AGE-albumin. This maybe because the different local sites of glycation or the residues which are most susceptible to glycation are positioned differently between these two enzymes that account for the different enzyme activities of Cathepsin B and D after exposure to AGEs. Further investigation should be done in the future.

The mechanisms of advanced glycation causing the observed dysfunction of enzyme could be due to either the enzyme active site residue being modified directly by Maillard chemistry and/or through secondary reaction with AGE-modified proteins phagocytised by lysosomal enzymes. Other studies in other proteins have found similar results that oxidised LDL can deactivate lysosomal proteases through forming covalent linkages with residues in the active site (Hoppe *et al* 1994). Furthermore, non-enzymatic glycation proteins from lens crystalline (Liang & Chylack 1984) and serum albumin (Shaklai *et al* 1984) have altered tertiary structure and function, while posttranslational modification of basic fibroblast growth factor by advanced glycation causes reduced activity (Giardino *et al* 1994). It is possible that the glycation in the local site of these three enzymes, Cathepsin D, Cathepsin B and acid phosphatase enzyme, are different and this could account for the different enzyme activity expression among of them which after exposed to AGEs.

Taken together, all these results may suggest that AGEs could have some influence on retinal pigment epithelial cell dysfunction through accumulation of components in the lysosomal, modifying enzymatic function and perhaps leading to abnormal degradative capacity. Further studies of the exact mechanism of the changes inside the lysosomes should be required to fully investigate this potentially important phenomenon.

4.1 Identification of Gene Expression of ARPE-19 Cells Exposed to AGEs/ALEs by Using RT-PCR

4.1.1 Introduction

As mentioned previously, advanced glycation end products have been implicated in a variety of age-related diseases, for example: Alzheimer's disease, cataract, and atherosclerosis. In J. T. Handa's (et al 1999) report, they quantified that there was an age-dependent increase in AGEs in human Bruch's membranes; the AGEs were identified in basal deposits and Drusen. In 2001, they also reported that AGEs altered mRNA phenotype in the ARPE-19 cells which induced by culturing the cells on growth factor reduced matrigel modified by AGEs (J.T. Handa et al 2001). The altered expressed gene clusters were included those involved in cell differentiation, maintenance of the basement membrane, and early apoptosis. These genes were previously not known to regulate by AGEs expressed in the RPE cells.

From their results, we hypothesize that AGEs may also induce ageing mRNA phenotypes in the RPE that would promote either up or down-regulation of gene expressions related to Cathepsin B, Cathepsin D and Acid Phosphatase genes in the RPE cells to match our previous experimental results. Initially, to address our hypothesis, we used the Real-time RT-PCR method to evaluate the gene expression of Cathepsin D, Cathepsin B and Acid Phosphatase gene expression after the APRE-19 cells were treated with and without glycation conditions in different time

scales feeding various concentrations of POS as in our previous study.

Real time RT-PCR is carried out by using the Lightcycler™ (Roche) system which is specialised to quantify gene expression. Many advantages have been found by using Real time RT-PCR. It includes greater specificity, sensitivity and speed. However the most significant advantage is that the results are quantitative. For example, using the Lightcycler system to quantify PCR, the reaction can be completed in less than 30 minutes. As Real-time PCR can constantly detect the product formation during the reaction, it will allow the products to be analysed at the optimal point. The conventional PCR can only analysis the end-point of the reaction (semi-quantitative).

The mechanism of The Lightcycler™ (Roche) is a micro-volume fluorimeter integrated with a rapid thermal cycler (Wittwer *et al* 1997a). It can continuously monitor the double strand specific dye SYBR green I, which allows us to analyze the PCR reaction in real time, and at the same time the product amplification is still in progress. Rapid thermal cycling is achieved by using air as the temperature transfer medium and the reaction is carried out in glass capillaries with a high surface to volume ratio (Wittwer *et al* 1997a). This quantitative PCR can be completed in less than 30 minutes due to these rapid thermal cycling properties (Wittwer *et al* 1997a). SYBR Green I have a special character; when it is unbound, the fluorescence level is very low but after binding to double stranded DNA, it will have a very strong fluorescence and can be measured after each cycle of product extension. Therefore, as the cycle numbers increase, the fluorescence increases. When it is denaturising, the dyed molecules are released and the fluorescence signal decreases.

The advantage of detecting products by using SYBR Green I is that there is

no sequence specificity. This will make it easier when adapting primers used for conventional PCR when used with the Lightcycler system (Morrison *et al* 1998). However, as it has no sequence specificity, the SYBR Green I will bind to any double stranded DNA which means the other non-specific double strand products will also be detected during the reaction, such as primer dimers. In order to overcome this problem, the fluorescence is measured at a temperature which is just below the product melting temperature (T_m) instead of at the elongation temperature to increase the sensitivity because only specific products are double stranded at this temperature point. Therefore it can eliminate detection of shorter, non-specific products such as primer dimers (Morrison *et al* 1998). The use of SYBR Green I for product detection has recently been successfully applied to measure retinal mRNA phenotype changes in the ARPE-19 cells which is induced by advanced glycation products (J.T.Handa et al., 2001)

In addition to the advantages of rapid thermal cycling properties, the Lightcycler also has the distinct advantage of product amplification and analysis which can occur at the same time. This is in contrast with conventional PCR, where product analysis is usually carried out separately from product amplification by agarose gel electrophoresis and visualisation using dyes such as ethidium bromide. However, the Lightcycler uses DNA melt curve analysis for product differentiation. The basis for melt curve analysis of PCR is the fact that each specific double-stranded DNA (dsDNA) has a specific melting temperature. The melting temperature is defined as the temperature at which 50% of the DNA is denatured and is determined by the product length and GC content.

The melt curve of DNA is generated by plotting fluorescence as a function of temperature, whilst the Lightcycler heats through the dissociation curve (Ririe *et al*

1997). The basic principle is that when the temperature increases, the fluorescence intensity gradually decreases until there is a sharp loss of fluorescence while the product is denatured, which means that a specific product melts at this temperature. The advantage of Lightcycler compared with Gel electrophoresis is that the melt curve analysis can distinguish different products with the same length but at different GC/AT ratio. For example, primer dimers or other non-specific amplification products can be seen to melt at lower temperatures in broader peaks. Due to its ability to tightly control temperature, the Lightcycler can precisely distinguish different PCR products based on their highly specific melting temperature. It can discriminate PCR products from each other that differ even within 2°C (Ririe *et al* 1997).

4.1.2 Materials & Methods

4.1.2.1 Sample Preparation

ARPE-19 Cell cultures and POS were prepared as described in section 2.2.1 and 2.2.2,

Briefly, confluent ARPE-19 cells were detached with trypsin-EDTA in 75cm^2 culture flasks and resuspened into Ham's F10 medium (10% FCS + 1% antibiotics and fungizone). After mixed well, the cells were transferred into 6-well plates which were either coated with glycated Matrigel or just plain plastic to reach confluence. During culturing time, the medium was changed every 3rd day until the cells were confluent. After confluence, the culture medium was changed to Ham's F10 medium (2% FCS + 1% antibiotics and fungi zone) for 24 hours. The Ham's F10 medium (2% FCS + 1% antibiotics and fungizone) was used continuously until the end of experiments.

POS were diluted with Ham F10 medium (2% FCS + 1% antibiotics) from stock concentration to 1×10^8/ml and 1×10^9/ml. For a negative control, only 4 ml of culture medium was added in each well (2wells). For 1×10^6/ml, 3.96ml of medium and 40µl of 1×10^8/ml were added in each well (2wells). For 1×10^7/ml, 3.96ml of medium and 40µl of 1×10^9/ml POS were added in each well (2wells). Medium with and without POS were changed every 48 hours until the end time point of the experiments (i.e. Day 1, 7, 14 and 28).

4.1.2.2 Total RNA Isolation

Total RNA was isolated with TRIzol reagent from all conditional treatments of ARPE-19 cell type using the manufacturer's protocol (Gibco BRL, Life technologies, UK). Briefly, the cells were lysed by adding 0.5ml of TRIzol

reagent to the monolayer and a cell scraper were used to detach the cells. The cells then were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. 0.2ml of Chloroform was added to the solution, vortexed and incubated at room temperature for 3 minutes. The mixture was separated by centrifugation at 12000*g* for 15min at 4°C. Following centrifugation, the solution forms three phases; the lower red phenol-chloroform phase contained proteins; the middle phase contained DNA and the upper aqueous phase contained the RNA. The aqueous phase was transferred to a fresh 0.5ml eppendorf tube and the RNA precipitated by mixing with isopropyl alcohol; the samples were centrifuged at 12000*g* for 10min at 4°C. At this point, the RNA pellet was washed with 75% ethanol and the sample was vortexed and centrifuged at 7500*g* for 5min at 4°C. The pellet was air dried for approx 5-10min and the RNA pellets were dissolved in 100μl of DEPC-treated water. The concentrations and purity of the RNA were determined by ultraviolet spectroscopy and by UV visualization on a denaturing formaldehyde-agarose gel. The newly extracted RNA was used immediately for RT-PCR analysis or frozen at -80°C.

4.1.2.3 Purity testing of Total RNA: Formaldehyde-Agarose Gel Electrophoresis and UV Transillumination

The overall qualities of the RNA samples were assessed via electrophoresis on a denaturing formaldehyde-agarose gel. The gel was prepared by dissolving 1% agarose in DEPC water and once cooled to 60°C, 0.01% of ethidium bromide (10mg/ml), Mops buffer (final concentrations of Mops 0.04M Mops, pH 7.0, 0.01M Sodium Acetate, 0.001M EDTA) and 37% formaldehyde (final concentration 6.6%) were added. The RNA (1μg) was denatured by heating at 60°C for 5min and then

mixed with loading dye (Promega, UK) in a 6:1 ratio. The RNA was electrophoresed using a Mops running buffer at 60V for 45min. After electrophoresis, the gel was visualized and digitally photographed on a UV transilluminator (UVi Tec, UK).

4.1.2.4 RT-PCR Amplification of Glycated RNA

Total RNA was used to synthesize cDNA using the manufacturer's protocol provided with a Reverse-iT™ 1st Strand Synthesis Kit (AB gene, UK) and performed in a thermo-cycler (DNA Engine, DYID, UK). Briefly, total RNA (1µg) was initially heated at 70°C for 5min in the presence of 1µl of anchored oligo dT (500ng); DEPC-treated water was added to give a total reaction volume of 13µl. The first strand cDNA synthesis reaction was performed in 40µl reactions composed of 4µl of first strand synthesis buffer, 2µl of dNTP mix (5mM) and 1µl of Reverse-iT RTase. The reaction was incubated at 47°C for 30min and terminated at 75°C for 10min. The newly synthesized cDNA were used immediately for PCR analysis/frozen at -20°C.

4.1.2.5 PCR Amplification of Glycated cDNA

PCR amplification of the cDNA were performed in a DNA thermal cycler (DNA Engine, DYID, UK). The PCR reaction mixture contained 1x PCR buffer (50mM Tris-HCL (pH 8.0)), 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 1% Triton X-100, $MgCl_2$, 100µM dNTPs, 100µM primers and 0.025U/µl Taq polymerase in a final volume of 40µl (all PCR reagents were purchased from Promega, UK). The primer pairs for the investigate gene fragments were shown in table 4.1.1 and were synthesized based on human sequences obtained from Genebank. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH),

assession number; BC023632. Acid phosphatase (APS), assession number;BT019589, Cathepsin B (CAB); assession number; NM147783), Cathepsin D (CAD); assession number BT020155.

All PCR primers were synthesized by MWG-Biotech, Germany. Table 4.1.1 showed the primer sequences, the accession numbers and optimized thermal cycling conditions used in these PCR amplification reactions. Negative controls of cDNA synthesis were carried out under the same experimental conditions, but in the absence of reverse transcriptase.

Table 4.1 PCR Primers and Parameters used for mRNA Quantity

Gene Product	Sequence	PCR Parameters	$MgCl_2$ (mM)
GAPDH (BC023632)	5-`TGATGACATCAAGAAGGTGCTGAA-3`(sense) 5-`TCCTTGGAGGCCATGTGGGCCCAT-3`(antisense)	75°C, 30s; 94°C, 2min; (94°C, 20s; 56°C, 30s; 72°C, 30s); 72°C, 5min (35 cycles)	**1.5**
APS (BT019589)	5-`GACAGCAAAGTCCAGGAAGC-3`(sense) 5-`GTGGTCTGATACGCGAGACA-3`(antisense)	75°C, 30s; 94°C, 2min; (94°C, 20s; 56°C, 30s; 72°C, 30s); 72°C, 5min (35 cycles)	**1.5**
CAB (NM147783)	5-`AGAATGGCACACCCTACTGG-3`(sense) 5-`TGCATTTCTACCCCGATCTC-3`(antisense)	75°C, 30s; 94°C, 2min; (94°C, 20s; 56°C, 30s; 72°C, 30s); 72°C, 5min (35 cycles)	**2.0**
CAD (BT020155)	5-`GACACAGGCACTTCCCTCAT-3`(sense) 5-`GGACAGCTTGTAGCCTTTGC-3`(antisense)	75°C, 30s; 94°C, 2min; (94°C, 20s; 56°C, 30s; 72°C, 30s); 72°C, 5min (35 cycles)	**2.0**

4.1.2.6 Optimization of Magnesium Concentrations and Annealing Temperatures for PCR Products

To ensure the successful amplification of the glycated gene fragments the optimum magnesium concentrations and annealing temperatures were determined. Firstly, the concentrations of $MgCl_2$ were investigated in increasing 0.5mM increments of 1.0mM, 1.5mM and 2.0mM. In parallel, the amplification of the

house keeping gene (GAPDH) at each $MgCl_2$ concentration was studied at the annealing temperatures of 50°C, 53°C and 56°C. The amplification of the lysosomal primer sets (APS, CAB and CAD) were optimized. The PCR and agarose gel electrophoresis were carried out as previously described. After visualization of the PCR products by means of UV trans-illumination the most robust amplification conditions were chosen for subsequent PCR studies.

4.1.2.7 Determination of Linear PCR Conditions

The linearity of the PCR reaction for all glycated gene fragments was established to ensure quantitative conditions. The RNA extraction and RT-PCR were carried out as previously described. The amount of PCR product was determined during increasing PCR cycle numbers in 3 cycle increments of 23, 26, 29, 31, 33 and 35. Following the PCR reaction agarose gel electrophoresis was performed and the gels analysed as previously described. The PCR cycle data were expressed graphically as the percentage increase in PCR product compared to 0 cycles at each cycle number. From the graphical plot a cycle number was determined which was in the linear range of the PCR reaction and therefore suitable for the subsequent lysosomal enzyme expression studies.

4.1.2.8 Real Time RT-PCR

The cDNA was amplified in a PCR utilising sequence-specific primers for the genes of interest (table 4.1). cDNA samples were diluted 1/10 and the reactions were performed in triplicate. The cDNA (2μl) was mixed with 0.5μM sequence-specific primers, 4mM $MgCl_2$ and Lightcycler Master Mix (Biogene), which contained deoxynucleotides and Taq DNA polymerise in reaction buffer so

that the final volume was 20µl.

The PCR reaction was performed in the Lightcycler (Roche Diagnostics Ltd, Lewes, UK). After an initial denaturising step at 95°C for 30 seconds, this was followed by 35 PCR cycles of denaturation at 95°C, then a primer-dependent annealing temperature for 3-5 seconds followed by elongation at 72°C for 4-9 seconds. The elongation period was determined by product length, with 1 second incubation for every 25bp of the product. Fluorescence was measured once per cycle after the product extension phase. For negative controls, one reaction was set up with the cDNA absent from the reaction mixture and a second negative control consisted of a cDNA sample that had undergone the reverse transcription reaction, but had the RT omitted (no RT control). In each series of PCR reactions, the standards used were dilutions of one of the samples (1:10, 1:25, 1:50, 1:100).

The product melting curves were obtained immediately after the PCR reaction by cooling the sample to 62°C, and then increasing the temperature slowly at increments of 1°C per second to 95°C whilst monitoring the fluorescence continually. To verify the specificity of the melt curve analysis PCR products were subsequently subjected to agarose gel electrophoresis, stained with ethidium bromide (10 µg/ml) and visualised on a TFX-20M UV Transilluminator (Gibco) connected to a digital camera (Kodak digital science).

4.2 Results

4.2.1 RNA Analysis

The purity of the RNA was tested by using agarose gel electrophoresis and it showed specific bands of ribosomal RNA. They were clearly visible and corresponded to the highly abundant 18S and 28S total ribosomal RNA. The integrity of these bands reflects the integrity of the mRNA. As both bands were sharp and clear without smearing, this indicated that the sample degradation was minimal. This was shown in Figure 4.1.

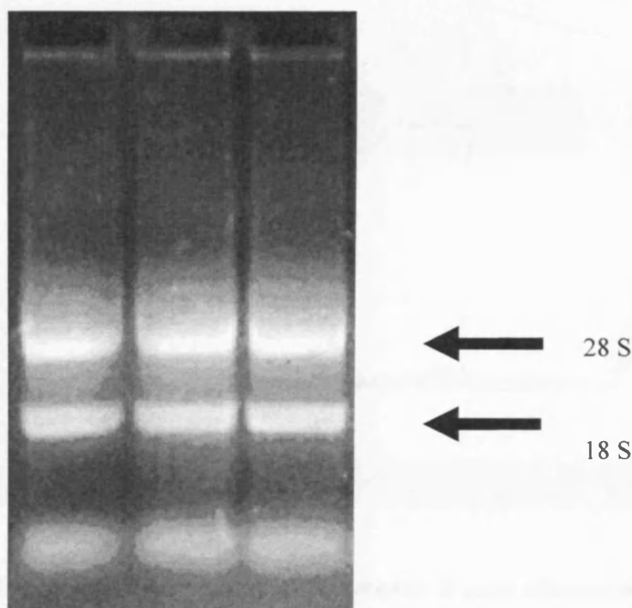


Figure 4.1 total RNA shows in agarose gel electrophoresis

Gel shows two 18s and 28s RNA bands, it indicates good quality of total RNA isolation for all samples as there was no smearing in the bands.

4.2.2 RT-PCR

The Lightcycler™ can provide two part of analysis of the data. One is

quantification and the other one is product analysis by DNA melting curves.

4.2.2.1 Product Analysis by DNA melt curves

The amplification reactions analysed by the melting curve showed a gradually deduction in fluorescence when the temperature was increased (Figure 4.2).

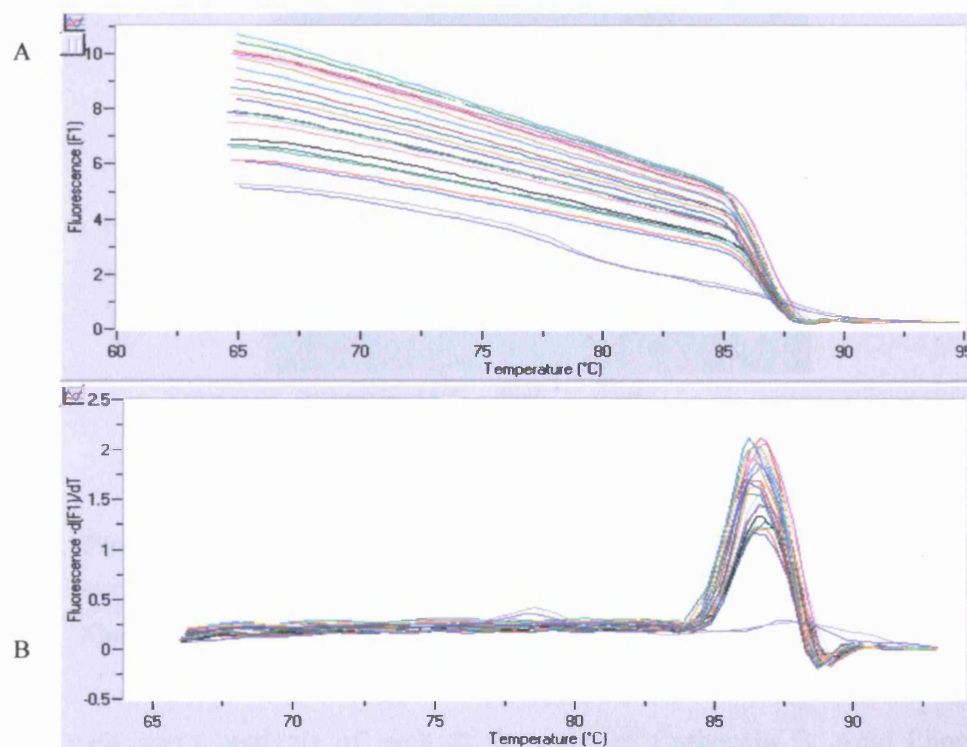


Figure 4.2 Melt curve analysis Cathepsin D gene expression by lightcycler.

When the product was denatured, the sharp drop in fluorescence occured which indicated the presence of a specific product that melted at this temperature. The fluorescence drop could also be seen more clearly as a peak in a first negative derivative plot (Figure 4.2b). This plot of the negative derivative of fluorescence with respect to temperature versus temperature (-dF/dT) produced melting peaks

rather than of a sharp fluorescence decrease (Figure 4.2b). This was also confirmed by agarose gel. For each primer pair used the presence of a single product was confirmed by identification of a single band at the predicted size by agarose gel electrophoresis. Figure 4.3 showed the presence of a single band at the correct size for the Cathepsin B, Cathepsin D and acid Phosphatase components.

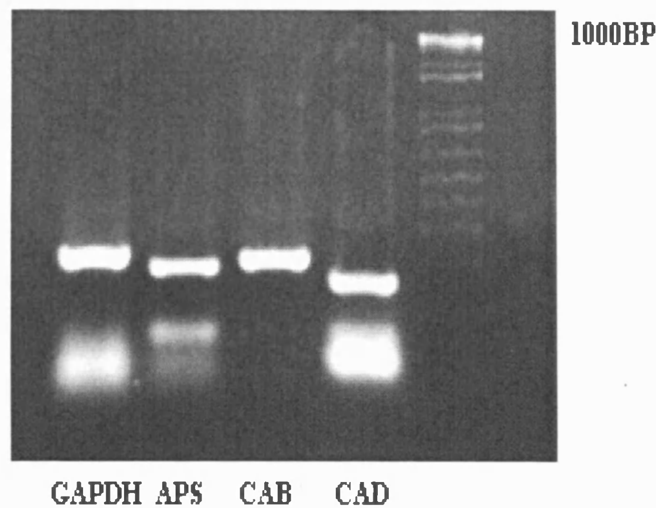


***Figure 4.3** Agarose gel electrophoresis showed the presence of a single band at the correct size for the GAPDH (240bps), Acid Phosphatase (195bps), Cathepsin B (212bps), and Cathepsin D (168bps) component.*

The melt curve analysis of each of the gene of Cathepsin B, Acid Phosphatase components and GAPDH had a single sharp peak indicating the presence of a specific product (Figure 4.4). Primer dimers can be seen to melt at lower temperatures and form broader peaks (Figure 4.4).

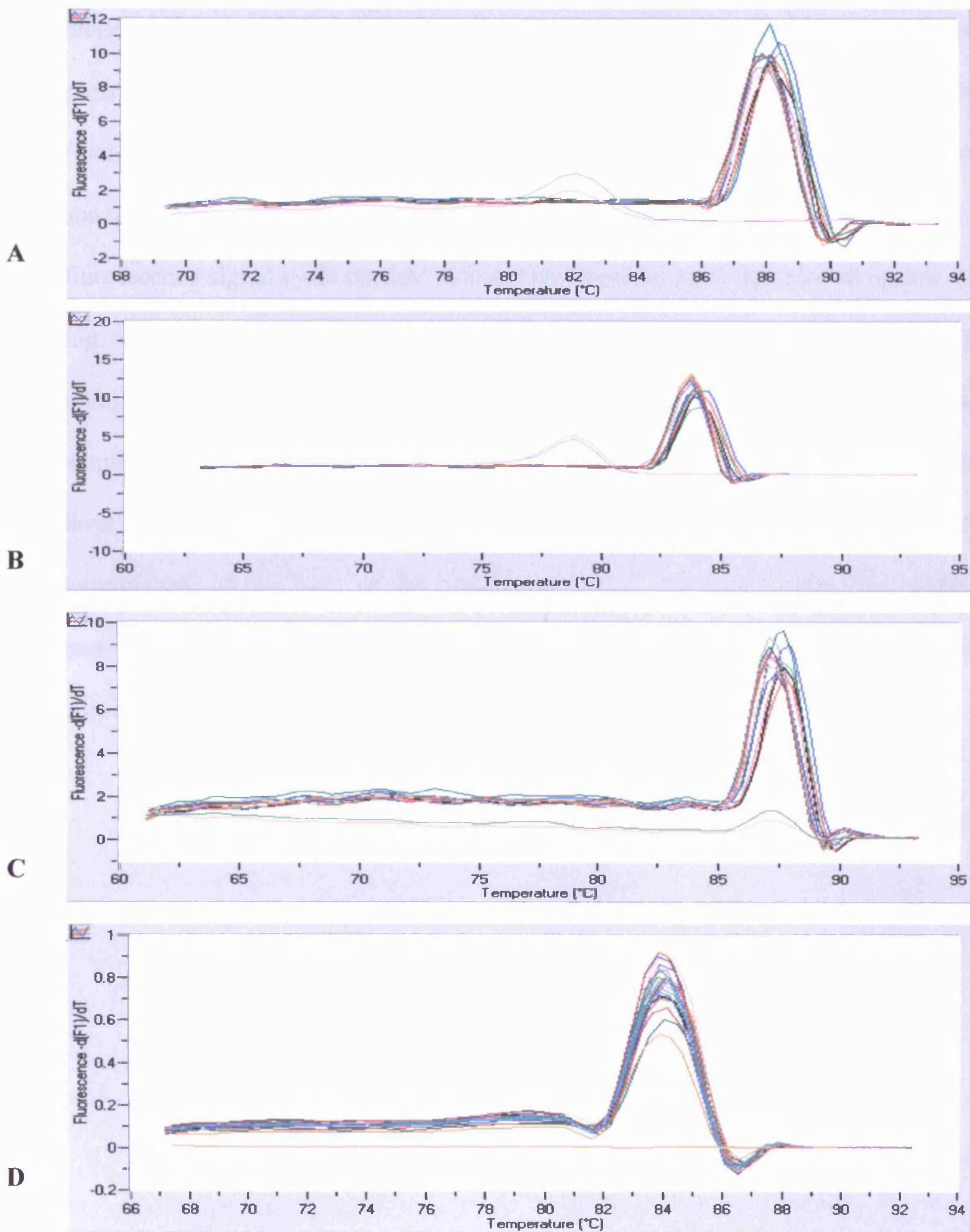


Figure 4.4 the melt curves for Cathepsin B (A), D (B), Acid Phosphatase(C) and GAPDH (D) house keeping genes.

4.2.2.2 Quantification

Figure 4.5 showed the light cycler quantification graphs. The exponential phase of the amplification reaction was identified as the "log-linear" portion in the plots of log fluorescence against cycle number (Figure 4.5a). A threshold level of the fluorescence from each amplification reaction was set in the log-linear region. The standard curve was created by using a control cDNA dilution series. The fluorescence signal cycle number crosses this threshold level was plotted against the log of the template concentration (Figure 4.5b) and this was used to define the relationship between template concentration and the cycle number where product amplification entered the log-linear region. Because the cycle number which the level of fluorescence raised above a background threshold value was inversely proportional to the long of the initial number of template copies, the relative concentration of message in each sample could then be derived from this standard curve (Figure 4.5b).

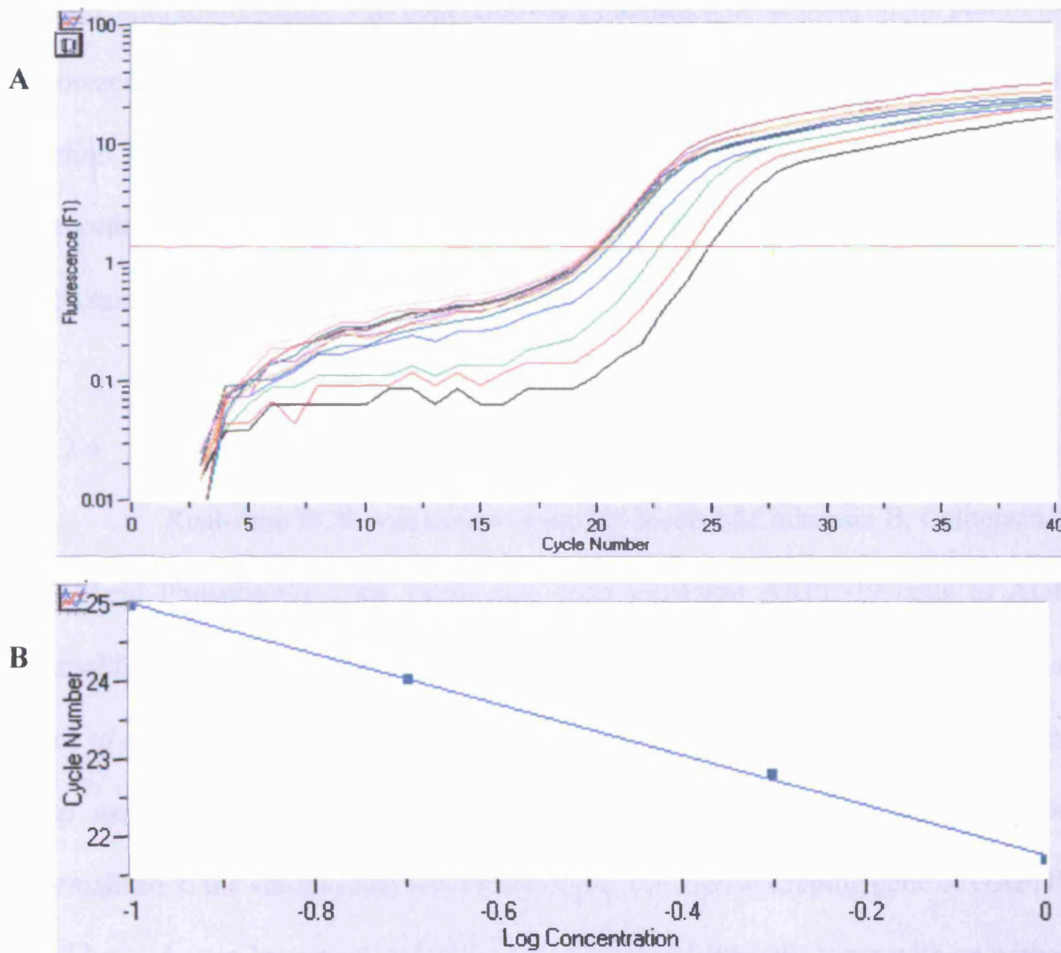


Figure 4.5 lighecycler Quantification of Cathepsin D gene expression

Photograph A showed logarithmic plot of fluorescence (F1) against cycle number. B showed the cycle number at which fluorescence crossed a threshold level was plotted against the log of known template concentration to produce a standard curve. The concentration of an unknown sample could be derived from its crossing point.

4.2.2.3 Results Data Analysis

The quantification and analysis of the product data was analysed by using the Lightcycler™ software. The fluorescence was measured once per cycle and it was displayed in real-time immediately after each measurement in a plot of fluorescence against cycle number, which was updated after each thermal cycle. For

quantification of data, the baseline of all reactions was first equalised and the background fluorescence was then removed by setting a noise band on the plot of log fluorescence against cycle number. The cycle number at which the log linear portion of each amplification curve intersected the noise band was inversely proportional to the log of the copy number (Figure 4.5) and relative expression in each sample was calculated from this graph.

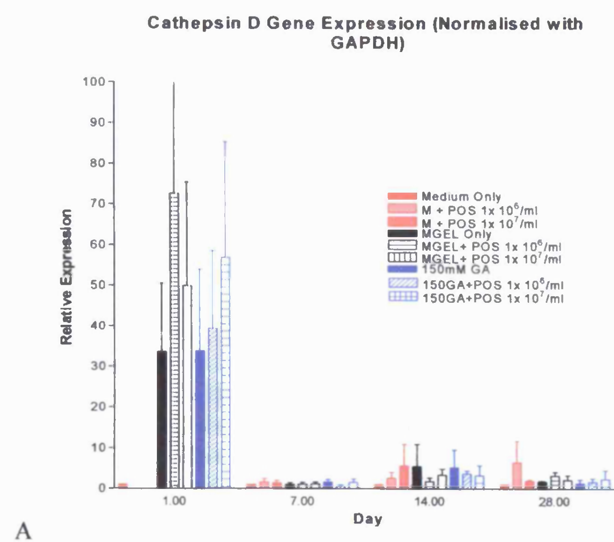
4.2.2.4 mRNA Expression in RPE After Exposure to AGE Modified Matrigel.

Real-time PCR was used to quantify levels of Cathepsin B, Cathepsin D, and Acid Phosphatase gene expression after exposure ARPE-19 cells to AGEs immobilised basement membrane extract (matrigel). mRNA expression levels of each of enzymes were shown in figure 4.6, expression levels of GAPDH RNA were also assessed and the mRNA levels of the individual components was then normalised to the values obtained for GAPDH. As a house keeping gene of GAPDH, it did not change by any circumstance, for example, the cells were with or without glycation during the experiments, the gene expression were expressed at the same levels from beginning to the end of experiments. This allowed it to be used for normalising the other genes' expression results.

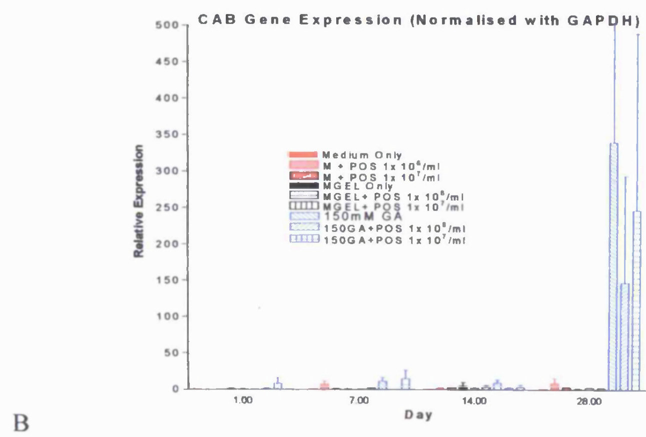
To allow analysis of gene expression between separate runs values were expressed as a percentage relative to normal. Statistical analysis was performed between the results obtained from the normal RPE cell by using a paired Student's t-test (2-tailed).

Results showed after 28 days cultures of ARPE-19 cells on glycated matrigel, the gene expressions of Cathepsin D, Cathepsin B and Acid Phosphatase of the cells were up and down regulated by AGEs treatment compared with the

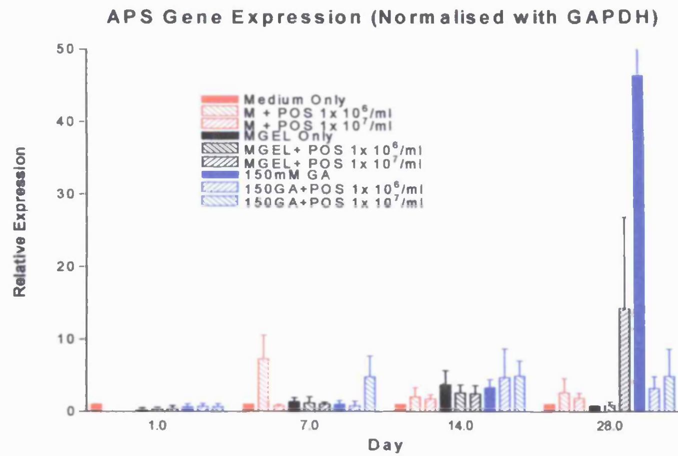
non-glycated treatment, which supported our results in chapter 3. Results showed in figure 4.6. for example, Cathepsin D activities were decreased 95.7% in cells cultured on 150mM GA matrigel fed with 1x10^7/ml POS compare with control; Cathepsin B activities were increased 984 folds, APS was increased 7.08 fold in the same conditions compared with Cathepsin D respectively.



A



B



C

Figure 4.6 ARPE 19 cell cultured on with and without glycated matrigel and relative gene expression of Cathepsin D (A) (It had [2.43/56.81] 95.7% decrease at cells treated with 150mM GA fed with $1x10^7$/ml POS compared with control); Cathepsin B (B) (It had [246/0.25] 984 fold increase at cells treated with 150mM GA fed with $1x10^7$/ml POS compared with control) and Acid Phosphatase (C) (It had [4.89/0.69] 7.08 folds increase at cells treated on 150mM GA fed with $1x10^7$/ml POS compare with control) were analysed by Real-time PCR.

4.3 Discussion

The formation of AGEs, such as CML and pentosidine is accelerated in regions of oxidative stress (Stitt et al., 1997). Age has been implicated in AMD's pathogenesis, accumulation in ageing and AMD retinas at pathologic sites in Bruch's membrane and the RPE (Stitt et al., 1998 &2000). AGE formation within extracellular matrices has been proposed to contribute to diminished barrier and filtration properties in Bruch's membrane. Glycosidation and lipoxidation reactions induced by oxidative stress have been linked to drusen formation and are proposed to contribute to the progression of AMD.

One of the most significant age-related changes in the RPE is the accumulation of lipofuscin. Each individual cell of the RPE is thought to internalize and degrade up to 200-4000 outer segment discs per day (Bok & Young 1969). Therefore it might have been expected to see changes in the expression of genes associated with the degradation of outer segment material, such as lysosomal enzymes.

I have shown for the first time that AGEs have a deleterious influence on the highly active and uniquely specialised lysosomal system in the RPE. However, it remains to be determined whether AGEs could be abnormal or subject to up or down regulation of the related lysosomal enzymes gene expression in the RPE under certain pathological conditions with ageing. J. T. Handa et al (1999) demonstrated that ARPE-19 cells in vitro grown on Growth Factor Reduced Matrigel modified by AGEs could induce an altered mRNA phenotype. They also found there were up regulated expression of AGE-receptors (Receptor 1 and Receptor 3) at the RNA and protein levels in the RPE cells compared to RPE

associated with morphologically normal Bruch's membrane which were related to AGEs exposure with ageing (Handa et al., 2001). The authors reasoned that genes relevant to a phenotype were tightly regulated and had constant expression, so that small expression changes would produce biologically important differences (Handa et al., 2001). This supposition had particular relevance when determining topographically related changes since we would expect to find small expression changes by regional location.

This suggested that AGEs might induce an ageing mRNA phenotype to the RPE that would promote the lysosomal enzyme gene expression and further to promoted degeneration of the RPE-Bruch's membrane complex which related with AMD in ageing. Cathepsin D was considered to be the main protease involved in the digestion of photoreceptor outer segment (Regan *et al* 1980). However, their expression in RPE had not been previously investigated. In the present study, as initial work to address our hypothesis, we used real time RT-PCR analysis method to investigate AGE modulation of the lysosomal enzymes gene expressions in the RPE. The RT-PCR approach allowed us to examine relative quantity changes of gene set, which uncovered an expression profile that could influence ageing of RPE cells. Our RT-PCR analysis indicated that expression levels of Cathepsin D was down-regulated after exposure to photoreceptor outer segment and AGEs (figure 4.6a). There was an exception that the CAD gene expressions affected by AGEs at day 7 had a dramatically decrease but compared with the enzyme activity in Chapter 3, it had a sharp increase. A new question is raised to clarify what is the inner mechanism regarding these differences and future study seems necessary.

Generally, the decreased of gene expression of CAD matched our previous results of the enzyme activity which decreased with ageing after 28 days treatment

with AGEs. However, it was inconsistent with a previous study that found Cathepsin D expression was not impaired by AGE treatment (Rakoczy et al., 1999) and that it was constitutively expressed at a high level in the RPE (Cavaney-Brooker & Rakoczy 1999).

It has been shown that Cathepsin B can participate in tumour invasion by degradation of extracellular matrix components (Tersariol et al., 2001). This can take place either intracellular by heterophagosomal activity of tumours cells (Sloane B.F. 1996) or extracellular by cell surface associated Cathepsin B (Sloane B.F. 1986). It has been demonstrated that the presence of Cathepsin B at plasma membrane results in focal dissolution of extracellular matrix proteins and enables the tumour cell to invade. From our results, it supported our hypothesis that Cathepsin B activity was increased and the gene expression was over expressed after AGE treatment compared with non-glycated condition in vitro, which might suggest it had an important role in retinal degeneration which could be related to AMD pathogenesis.

Acid phosphatases are widely found in nature but normally at a low concentration in humans. Although they have similar function, APS differ widely regarding tissue and chromosomal origin, molecular weight, amino acid homology, sequence length, and resistance to L(+) tartrate and to fluoride. Nadler and Egan (1970) reported deficiency of total lysosomal acid Phosphatase was an autosomal recessive disorder. Over expression of APS in man was also found to be related to a pathophysiological process for example in prostate cancer, Gaucher's disease-an inborn error of cerebroside metabolism that was inherited as a recessive disorder etc. In this study, Acid Phosphatase was over expressed especially after AGEs treatment, this work addressed the hypothesis.

A glimpse at the diversity of gene expression alterations induced by AGEs was made possible with a high throughput approach. Further work characterizing AGE-induced changes, determining what changes were "causes or effects," and identifying an AGE mediated ageing effect to the RPE *in vivo* is warranted and underway. In our results, although the fold changes induced by AGEs were moderate before 14 days but were considerable higher after 28 days treatment at the end, we confirmed the expression patterns of gene expression by RT-PCR. This analysis identified genes that were previously not known to be regulated by AGEs, expressed by RPE cells, or implicated in ageing changes.

Further in depth studies are required before speculating if there is any functional change associated with the differentially expressed genes. However, the large number of differentially expressed genes emphasizes the difficulty of interpreting results based on a single cell line and indicates the need for additional study before making suggestions about what may happen *in vivo.* Caution must be used in fully comparing the AGE-induced gene expression profile found in this study to ageing of the RPE or AMD. Although the ARPE-19cell line has been characterized in depth and displays many of the characteristics of the RPE *in vivo*, making generalizing statements referring to the *in vivo* situation, which presumably can only be justified after the study of multiple cell lines or even in primary cells.

We acknowledge that matrigel, the best-known basement membrane approximation *in vitro* could induce artificial gene expression changes or that nonphysiologic AGEs could be produced by glycolaldehyde and to be used for *in vitro* research purposes.

5.1 GENERAL DISCUSSION AND CONCLUSIONS

Ageing is considered to be one of the most important risk factors for the development of AMD. It has been defined as "the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age" (Harman 1981). The accumulation of lipofuscin in the RPE is one of the major markers of ageing in the retina. Retinal pigment epithelial lipofuscin originates largely from the shed tips of photoreceptor outer segments, and it begins to accumulate in retina since childhood (Katz *et al.,* 1986; Boulton *et al.,* 1991). They are phagocytized throughout life, producing an incompletely degraded residuum that accumulates in the end stage lysosomes of RPE (Feeney 1978). This is reflected by increasing accumulation of auto-fluorescent granules in the RPE cell. They are considered to represent lipofuscin derived from autophagy developed in several types of cultured cells (Gao *et al.,* 1994). Incomplete degradation of POS is thought to contribute to RPE lipofuscin accumulation (Boulton *et al.*, 1989).

Maintenance of RPE cultures at confluence and feeding with POS has been identified as a useful model to identify the ageing process in the RPE *in vitro.* These issues were addressed in our results both in the ARPE-19 cell line and primary cells from aged donors. From our results, the accumulation of auto-fluorescent granules increased moderately after 28 days culture without feeding POS but showed a dramatically increase in the cells feeding with highest POS ($p=0.0081$). Results for primary cells showed the similar trend. It was also shown the changes had no significant gender differences. Unfortunately, during this time,

it was impossible to get primary cells from younger donors. We could not confirm the hypothesis that if the age variance had a key role in the accumulation of auto-fluorescent granules in the RPE; future work should address this issue depending on donor's availability.

A great amount of evidence has shown that advanced glycation can also play a considerable role in age-related RPE dysfunction (Kasper *et al* 1999; Sebekova *et al.*, 1998). Because the accumulation of advanced glycation end-products in the RPE has been addressed, it is a normal process of ageing. It is possible that any interfering effects which increase the accumulation of AGEs at the RPE-Bruch's-choriocapillaris interface during ageing may contribute or facilitate the development of AMD and relate degenerative disorders in the ageing patient.

It is well known that AGEs can mediate a wide range of cellular responses, such as increased deposition of lipofuscin within RPE; altered RPE cell lysosomal enzymes activities; reduced cell phagocytosis ability; changed the cell's oxidative stress ability (Boulton *et al.*, 1998) and altered ageing mRNA phenotype (Handa *et al.*, 2001). Each of these responses induced by AGEs could be considered as an important component, which could contribute to age-related RPE dysfunction. Therefore, we assessed each of these AGE effects individually.

It was hypothesised that the advanced glycation may be involved with the pathogenesis of age-related dysfunction of the Retinal Pigment Epithelial; for example, age-related macular degeneration-AMD. This hypothesis was developed from previous findings that showed AGEs were present in drusen and accumulated in Bruch's membrane during ageing (Handa *et al* 1995, Ishibashi *et al* 1998). On

the other hand, there were a few *in vitro* studies had demonstrated that exposure of the RPE to AGEs had shown a range of latent important pathogenic responses (Lu *et al* 1998, Handa *et al* 1998, Honda *et al* 2001). Therefore, to investigate the effects of advance glycation end-products on retinal pigment epithelial cell layer *in vitro* is a relevant and timely approach to understand how it affects the RPE with ageing. It will also help us to understand many other important age-related diseases for further research of this pathogenic mechanism within the context of RPE ageing. This may lead to exploration of a novel method for the future therapy.

In order to quantify the overall effect of the non-enzymatic glycation on RPE function, we evaluated the effect of AGE exposure at a molecular and cellular level. The cellular level investigations involved *in vitro* assays, such as exposure of the RPE (cell line and primary cells from donors) to AGEs to bring about the damaging effects to induce the different abnormal cellular responses. Therefore, to investigate this issue, the RPE monolayer was grown on AGE-modified basement membrane extract (matrigel ™ substrate) formed via 50-200mM glycolaldehyde treatment for up to 28days. This approach is of proven value within *in vitro* systems and represents an excellent model of the aged, cross-linked extracellular matrix (Paul R.G, Bailey A.J. 1996, Xamamoto H. 1997).

There were significant increases of accumulation of auto-fluorescence granules within the RPE were observed in our experiments, which were manifested in two ways. Firstly, there were significant morphological changes observed under a light microscope, especially after cells exposed to the AGE modified matrigel after 28 days culture. We believe this is the first time that the

visible changes of RPE cells under ageing conditions with glycation treatment have been observed. Secondly, flow cytometry analysis also revealed these ageing changes and they were significant induced by AGEs. It appeared to be consistent with a recent preliminary report that indicated greater auto-fluorescence in human RPE that were brought to replicative senescence *in vitro* (Cabral *et al.,* 1990). However, this outcome appeared to conflict with findings in bovine RPE; this might be due to species differences (Skumatz *et al.*, 1998). Older human donors' RPE cells were also used in the current study and showed similar results compared with the cell line. Donor age variance in this study could not be tested to monitor the affect on the development of auto-fluorescence *in vitro* because there were no young donors' RPE cells available.

In addition to the effect of AGEs on RPE cell dynamics, it was hypothesized that AGEs could be trafficked to the lysosomes where they could affect RPE function, possibly through accumulation in lysosomal compartments leading to a reduction in derivative capacity and then possibly leading to lipofuscin formation. Incomplete proteolysis in lysosomes was thought to contribute to lipofuscin accumulation in RPE in situ (Feeney 1978). We have demonstrated that lipofuscin is capable of inhibiting lysosomal enzyme activity in both biochemical assays (Boulton *et al.,* 1998) and using *in vitro* models (Boulton *et al.,* 2001). RPE accumulation of lipofuscin and dysfunction of lysosomal degradative capacity may be related to AGE formation *in vivo.* Kasper et al., have demonstrated AGE-mediated dysfunction of Cathepsin D expression and enzymatic activity in lung epithelium (Kasper *et al.,* 1999) and Cathepsin B, L and

H also show reduced proteolytic activity after exposure of kidney epithelial cells to AGE-albumin (Sebekova *et al.,* 1998).

We therefore considered the possibility that AGEs/ALEs may have a key role in the formation of auto-fluorescent inclusion in the RPE, and may result from reduced lysosomal activity *in vitro*, causing the accumulation of autophagic materials. This is important because the primary function of the RPE is to phagocytose and degrade POS. We selected the aspartic proteinase Cathepsin D as a marker enzyme of lysosome because it is widely regarded as the most important protease in this process (Regan *et al.,* 1980); Cathepsin D also has a high activity in RPE cells. This enzyme has already been implicated in age-related dysfunction (Boulton *et al.,* 1994).

From our results, Cathepsin D activities increased during the first 14 days of treatment after confluence and this was contradicted with previous reports. From 14-28days culture, the enzyme activities showed a significant decrease. This was consistent with the report of Boulton (*et al.,* 2001). The important issue from this study was that the decrease did not show glycation concentration dependence, but was time and POS-dose dependent. It could be possible that the AGE concentration scales used in this study may be too high even at the lowest scale (50mM); low glycation conditions are suggested for the future study. Also the RPE cells could possibly produce auto-fluorescent inclusions as they digest material. As they eat up the matrix if there were lysine reactions occurring with free aldehydes intermediates and e.g. lysines in the matrix, the auto-fluorescent granules can be formed so they are called lipofuscin like fluorophores. There shouldn't be much, only loads more with the glycated matrices.

Results were also confirmed using primary cells from donors. This may also suggest that increased lysosomal enzyme activity may help prevent accumulation of auto-fluorescent materials in growing cells, which could lead to a new therapeutic method for AMD.

Cathepsin B activities were also studied, results showed a decrease for the first 14 days but an increase after 14 days to reach a peak at 28 days. The changes were glycation concentration and POS-dose dependent. It had a difference compared with the report by Sebekova et al., (Sebekova *et al.*, 1998). APS activities had a big variation during the different culture time. Without glycation, the enzyme activity finally increased after 28 days feeding with POS, which was in contradiction with previous results (Sebekova *et al.*, 1998). This may be because the time period used in our experiments was longer. The APS activities showed a significant decrease at 28 days after glycation treatment (50mM GA feeding $1x10^7$/ml POS, p=0.0021 compare with control). As there were so many reports regarding the APS activity difference *in vitro*, further research may be necessary to be performed in different cell types. Having investigated the effects of AGEs on Cathepsin D, B and Acid Phosphatase activity, further studies should also concentrate on the many other RPE lysosomal enzymes.

Ageing is a complex phenomenon. It depends on the interaction among of numerous genes, cellular pathways and environmental risk factors. Real time RT-PCR has been developed to use for analysis changes in patterns in the gene expression of ageing retina in a few patients (Yoshida *et al.*, 2002). It is important to determine whether there is a genetic basis for ageing associated retinal diseases such as AMD, via a hypothesis-independent profile. Several genes involved in cell

growth and protein processing are preferentially expressed in the retina of young patients, where as genes involved in stress responses and energy metabolism play key roles in retinal ageing (Yoshida *et al.,* 2002). The analysis of gene expression in pathological ageing tissue by using of real time RT-PCR may lead to the identification of specific genes responsible for these diseases.

Previous studies have demonstrated that AGEs can influence the behaviour of RPE cells *in vitro* by inducing the expression of abnormally high levels of the growth factors PDGF-B and VEGF (Handa *et al* 1998, Lu *et al* 1998) and mRNA phenotypes (Handa *et al.,* 2001). We hypothesized that AGEs could also influence cell dysfunction via tertiary modification of essential gene expressions of lysosomal enzymes, which are important for regulating cell responses such as phagocytosis of POS and maintains the RPE normal function.

Results from this aspect of the study have found that AGE-modification of the expression of genes significantly affects RPE behaviour, in terms of impaired phagocytosis and maintenance of RPE. The fold changes in CAB, CAD and APS were dramatic. For example, the cells treated with 150mM glycation concentration feeding with $1x10^7$/ml POS, showed gene expression changes in CAB 700 fold greater than control; in CAD, it was a 70% decrease and it was 12 times higher than control for APS activities. Since Bruch's membrane is known to accumulate AGEs and become increasingly crosslinked with age (Handa *et al.* 1999; Farboud *et al.* 1999; Verijl *et al.* 1998), AGEs may therefore have an important role inducing RPE dysfunction with implications for the pathogenesis of many RPE-related disorders.

The pathological ageing of the retina is a complex phenomenon that should benefit from therapeutic interventions such as the modification of risk factors (for example antioxidant oral supplements for AMD patients) to prevent disease progression, and the modification of intermediated disease mechanisms. We originally proposed that AGEs could play a very important role in the age-related dysfunction of the retinal pigment epithelial in humans. In our conclusion, it had been shown that there was some evidence from different methods of *in vitro* study to indicate that AGEs appear to contribute to RPE dysfunction with ageing. In summary, it has been demonstrated that the molecular and cellular function of the RPE could be transformed by non-enzymatic glycation. All these results supported the fact that AGEs had a considerable vital role in age-related RPE dysfunction. However, it was not possible to completely address the hypothesis that AGEs were responsible for the development of age-related disease in the RPE.

Of course, although AGEs may not have contributed to the cause of age-related retinal disease, they certainly promoted the progression of age-related retinal disease. This will help us to develop a new strategy to investigate new drugs to prevent or decrease the accumulation of AGEs in the outer retina, which will restrict the dysfunction of RPE in ageing patients. To date there are many drugs, such as AGE inhibitors and cross linking breakers under development and in use for a wide range of diabetes and age-related disorders. However, there is relatively little attention given toward them for a potential use in the eye related ageing dysfunction field of research. Further research in the future would help to promote a few new therapeutic choices for treatment or take precautions against age related macular degeneration and age-related outer retina dysfunction.

Appendix I: materials and suppliers

Acetone	Sigma-Aldrich
Acetic acid	Acros Organics
Agrose powder	Sigma
Ammonium hydroxide	Sigma-Aldrich
Benzylpenicillin	Sigma Chemical Co
BCA-Kit	Pierce Biotechnology
Bovine serum albumin	Gibco
Citric acid	Acros Organics
Chloroform	Sigma Chemical Co
Diethylpyrocarbonate (DEPC)	Sigma chemical Co.
Dimethyl sulphoxide	Sigma Chemical Co
DL-Tyrosine	Sigma-Aldrich
EDTA	Sigma Chemical Co
Ethanol	Sigma-Aldrich
Ethidium bromide	Sigma
Fetal calf serum	TCS Biological Ltd
Formaldehyde	Sigma
Fungizone	Sigma Chemical Co
Glycerol	Sigma
Glutamine	BDH Laboratory Supplies
Glycoaldehyde	Sigma-Aldrich
Haemoglobin	Sigma-Aldrich
Ham's F10 growth medium	Gibco BRL(life Technologies Ltd.)
HEPES buffer solution	Gibco

Hydrochloric acid	BDH Laboratory Supplies
Isopropanol	Sigma
Magnesium chloride	BDH Laboratory Supplies
Matrigel	Becton-Dickinson
Methanol	Sigma Chemical Co
MOP powder	Sigma
Penicillin powder	Sigma
p-Nitrophenylphosphate	Sigma
PBS Buffer tablets	Sigma
Pepstatin	Sigma
Potassium chloride	BDH Laboratory Supplies
Potassium phosphate	Acros Organics
Sodium bicarbonate	BDH
Sodium Borohydride	Sigma-Aldrich
Sodium chloride	BDH Laboratory Supplies
Sodium citrate	Sigma
Sodium Formate	BDH Laboratory supplies
Sodium hydroxide	BDH Laboratory Supplies
Sodium phosphate	BDH Laboratory Supplies
Streptomycin sulphate	Sigma Chemical Co
Sucrose	BDH Laboratory Supplies
TCA	BDH Laboratory Supplies
Tris-acetate	Sigma Chemical Co
Trypsin	Sigma
Triton X-100	Sigma Chemical Co

Trypsin	Sigma Chemical Co

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