**Title: Mitochondrial Dysfunction and Oxidative Stress in Corneal Disease**

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Abbreviations: BCL-2: B-cell Lymphoma protein, TEM: Transmission electron microscopy, CEC: Corneal endothelial cells, ROS: Reactive oxygen species, RNS: Reactive nitrogen species, UV: Ultraviolet, Nrf-2: Nuclear erythroid factor 2, H2O2: Hydrogen Peroxide, mtDNA: mitochondrial DNA, SOD: Superoxide dismutase, NADPH: Nicotinamide adenine dinucleotide phosphate, ALDH3A1: aldehyde dehydrogenase 3, MT3: metallothionein 3, TXNRD1: thioredoxin reductase 1, FECD: Fuchs endothelial corneal dystrophy, OCT: Optical coherence tomography, NO: nitric oxide, eNOS: endothelial nitric oxide synthase, MMP-2: Matrix metalloproteinase2, TIMP-1: Tissue inhibitors of matrix metalloproteinase.

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**Abstract**

The cornea is the anterior transparent surface and the main refracting structure of the eye. Mitochondrial dysfunction and oxidative stress are implicated in the pathogenesis of inherited (e.g. Kearns Sayre Syndrome) and acquired corneal diseases (e.g. keratoconus and Fuchs endothelial corneal dystrophy). Both antioxidants and reactive oxygen species are found in the healthy cornea. There is increasing evidence of imbalance in the oxidative balance and mitochondrial function in the cornea in disease states. The cornea is vulnerable to mitochondrial dysfunction and oxidative stress due to its highly exposed position to ultraviolet radiation and high oxygen tension. The corneal endothelium is vulnerable to accumulating mitochondrial DNA (mtDNA) damage due to the post- mitotic nature of endothelial cells, yet their mitochondrial genome is continually replicating and mtDNA mutations can develop and accumulate with age. The unique physiology of the cornea predisposes this structure to oxidative damage, and there is interplay between inherited and acquired mitochondrial dysfunction, oxidative damage and a number of corneal diseases. By targeting mitochondrial dysfunction in corneal disease, emerging treatments may prevent or reduce visual loss.

1. **Introduction**

The eye is a highly specialized organ of photoreception, an optical system able to focus the light energy from the environment on to the retina, which is the receptor of the visual pathway. The cornea, the anterior transparent window of the eye, is a crucial part of this optical system, creating 80% of the refractive power of the eye. The cornea is a dome-shaped transparent structure, and its shape and clarity, are the main characteristics enabling such great refractive power. The cornea, being avascular, obtains its nutrients from the tear film, the aqueous humour and blood vessels at the peripheral edge of the cornea. Human corneal transparency is the result of a number of related factors: avascularity, structural regularity of the covering epithelium, regular arrangement of the extracellular and cellular components in the stroma and functionality of the endothelium to regulate corneal hydration (Nita and Grzybowski, 2016; Zierhut et al., 2008). The normal corneal structure comprises of five well-defined layers from the external to internal corneal surface: the epithelium (multilayer), Bowman’s layer, stroma (interlaced with keratocytes), the Descemet membrane and the endothelium (monolayer) (Figure 1). Even small malfunctions or malformations in any of these components and/or an impaired communication between them can compromise their function. The unique physiology of the cornea predisposes this structure to oxidative damage, and there is interplay between inherited and acquired mitochondrial dysfunction, oxidative damage and a number of corneal diseases.

Mitochondrial mutations and dysfunction has been implicated in other ocular conditions. The most common mitochondrial disease is Leber’s hereditary optic neuropathy (LHON). (Chinnery et al., 2001) This results in the degeneration of retinal ganglion cells and a progressive degeneration of the optic nerve. (Jankauskaitė et al., 2016) About 70% of all LHON cases are caused by the 1178G>A mutation of the mitochondrial deoxyribonucleic acid (mtDNA).(Cwerman-Thibault et al., 2014) There is an increasing body of evidence from genetic studies that mtDNA mutations and subsequent dysfunction may contribute to the pathogenesis of other debilitating ocular conditions including glaucoma (Lascaratos et al., 2012; Sundaresan et al., 2014) and age related macular degeneration (AMD). (Terluk et al., 2015) Also rarely oncocytomas (or oncocytic adenomas) may arise in the simple or glandular epithelia of the ocular adnexa. (Jones et al., 2016) In this condition these cells demonstrate an eosinophilic appearance and excessive and abnormal mitochondria content. (Østergaard et al., 2011)

**Figure 1- Histological section of the cornea stained with haematoxylin and eosin stain demonstrating the five layers of a normal human cornea (E- Epithelium, BL- Bowman’s layer, S- Stroma, DM- Descemet’s Membrane, EC- Endothelial cells).**



E

S

DM

BL

EC

1. **Mitochondrial function in the healthy cornea**

Mitochondria have a functional genome separate from that of nuclear DNA.(Leonard and Schapira, 2000) The human mitochondrial genome is 16,569bp long and forms a closed circular molecule. (Anderson et al., 1981) Human mitochondrial DNA contains 37 genes, all of which are essential for normal mitochondrial function. Thirteen of these genes encode enzymes, which are crucial for the oxidative phosphorylation pathway required for the production of the majority of cellular adenosine triphosphate (ATP).(Lascaratos et al., 2012)

There are three main cell types in the cornea with have specific structural and functional roles: corneal epithelium cells, keratocytes in the corneal stroma and a highly-specialised corneal endothelium. The corneal epithelium is stratified (consisting of five or seven cell layers) and its central thickness is approximately 50-52 μm (Hogan et al., 1971). The corneal epithelium is metabolically highly active, especially the deepest basal epithelial cells, which support the complete turnover of the epithelium over 5 to 7 days (Hanna and O’Brien, 1960). Despite this high turnover rate, the epithelium must maintain the same thickness profile over time to maintain corneal power and refraction. The epithelial thickness profile can affect the total corneal power because it determines the shape of the air-tear film interface, but also because of the difference in refractive index between the epithelium and the stroma (Patel et al., 1995). Mitochondria provide energy for cellular activities and play an essential role in apoptotic signal transduction in the corneal epithelium. The normal function and energy production in the corneal epithelium depends on the action of both pro- and anti-apoptotic B-cell lymphoma (BCL-2) family proteins and their interaction at the mitochondrial membrane (Hazlett, 2007; Lim et al., 2009; Niswander and Dokas, 2007; Szegezdi et al., 2009). Mitochondrial damage has been implicated in corneal epithelial cell death in dry eye disease, where hyperosmolarity (a state seen in dry eye disease) induces apoptosis of human corneal epithelial cells through cytochrome c mediated death pathway (Luo et al., 2004).

In comparison to the epithelium, the remainder of the corneal cells have a slow turnover rate therefore increasing its susceptibility to damage by oxidative processes (Zierhut et al., 2008). The stroma, the major component (about 90%) of the cornea, is a collagenous tissue composed of multiple lamellae of tightly packed parallel collagen fibrils and keratocytes. Uniform stromal collagen diameter and orderly packing is essential for tissue transparency. The keratocytes (3% of the stromal volume) remain quiescent throughout adult life (Pinnamaneni and Funderburgh, 2012). Transmission electron microscopy (TEM) has shown that anterior stromal keratocytes contain twice the number of mitochondria as the posterior two-thirds of the stroma, which correlates with the higher oxygen tension and cell density of the anterior stroma (Kaufman et al., 2011; Muller et al., 1995; Snyder et al., 1998).

The corneal endothelium is a monolayer of hexagonal cells, which has a critical role in maintaining corneal hydration and thus transparency. Corneal endothelial cells (CECs) are highly inter-digitated and possess apical junctional complexes that, together with abundant cytoplasmic organelles including mitochondria, are indicative of their crucial role in active fluid transport (Zavala et al., 2013). In order to maintain the corneal transparency, active transport of water out of the corneal stroma and endothelium into the anterior chamber is required. The corneal endothelium has a high rate of metabolic activity to regulate corneal hydration by active transport in the corneal endothelium (Nita and Grzybowski, 2016). Histologically, the corneal endothelium cells contain a large nucleus, a prominent endoplasmic reticulum and a large number of mitochondria, which provide the high amount of ATP necessary for the endothelial Na+/K+ ATPase active transport pump (Laing et al., 1992; Yu et al., 2011).

The corneal endothelial cell count declines throughout life with an estimated rate of decline of 0.6% per year (Bourne et al., 1997); the endothelial cell density of 3 to 6 year old children is 4000 to 3500 cells per mm2 (McCarey, 1979), whereas middle aged adults (30 years of age) can have a range between 2700 to 2900 cells per mm2, and adults >75 years of age can have a range of endothelial cell densities between 2400 to 2600 cells per mm2 (Hoffer, 1979; McCarey, 1979; Yee et al., 1997). The endothelium is the corneal cell layer with the lowest mitotic activity and there is no evidence that human endothelial cells divide under normal circumstances (Bourne, 2003). Damage to the endothelium is therefore clinically and functionally more significant than damage to other corneal layers, as this can result in irreversible cell loss and subsequent loss of visual and corneal function (Zavala et al., 2013). Oxidative stress has been shown to cause corneal endothelial cell death by apoptosis or necrosis (Cho et al., 1999; Hull and Green, 1989). The corneal endothelium can be subjected to a number of different stressors which can accelerate the normal enlargement and loss of cells with age e.g. intraocular surgery (DB et al., 1985; Matsuda et al., 1984; Olsen, 1979), endothelial wounds (Landshman et al., 1989; Yee et al., 1987), ocular (Brooks and Gillies, 2016; Olsen, 1979; Setala, 1979) and systemic diseases e.g. diabetes (Schultz et al., 1984). Reactive oxygen species (ROS) generated from ultrasonic energy (Takahashi, 2005)(specifically from phacoemulsification in cataract surgery) may be a major mechanism causing the subsequent endothelial cell damage and failure.

1. **Oxidative stress and the cornea**

The cornea, given its highly exposed position, receives a significant amount of high-tension atmospheric oxygen and sunlight, including the ultraviolet range. These factors result in the generation of ROS and subsequent oxidative stress in the cornea (Wenk et al., 2001). Oxidative stress in the cornea is a consequence of an imbalance between Reactive oxygen species (ROS) production and the antioxidant capacity of the corneal cells (Choi et al., 2011). Reactive nitrogen species (RNS), especially those containing oxygen, may also contribute to oxidative stress. ROS are important in cellular homeostasis (Knapp and Klann, 2002) and cells require a specific balance of ROS and RNS for normal cellular physiological functions such as cell growth, proliferation, differentiation and apoptosis (Knapp and Klann, 2002). When the levels of ROS/RNS are excessive there is a detrimental influence on cellular physiology (Droge, 2002; Finkel and Holbrook, 2000). The excessive accumulation of ROS or RNS in the cornea affects signal transduction, cellular proliferation and promotes cell death (Wojcik et al., 2013). Xanthine oxidase, an enzyme known to generate ROS, is present in human corneal epithelium and endothelium and may contribute to oxidative stress (Čejková et al., 2002). The cornea is also equipped with an antioxidant defence system to counteract oxidative stress which consists of a number of molecules which can counteract free radicals: low molecular weight antioxidants (vitamin C, vitamin E, β-carotene, reduced glutathione, and ferritin) and high molecular weight antioxidants (catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase). In ocular disease, as well as in the case of several neurodegenerative diseases, there is a normal decline of antioxidant defence mechanisms with age, which increases the vulnerability of cells to oxidative damage (Finkel and Holbrook, 2000).

One of the functions of the cornea is to absorb ultraviolet (UV)-light entering the eye and hence protect the retina and lens from these high wavelengths of potentially damaging light (Ringvold, 1980; Zierhut et al., 2008). The majority of UV-A light (315-400nm) is absorbed by the lens whereas UV-B (280-315nm) is absorbed by the cornea (Wojcik et al., 2013). UV-A radiation causes apoptosis in corneal epithelial cells by regulating the expression of antiapoptotic BCL-2 (B-Cell lymphoma 2) proteins (Matalia et al., 2012). Apoptopic stimuli (including UV-radiation) promote the release of cytochrome c from the mitochondria (Hull and Green, 1989; Tournier et al., 2000) and activate downstream destruction pathways including the caspase cascade (Luo et al., 2007). The transcription factors nuclear factor erythroid 2 (Nrf2) and p53 are involved in the stress response in the cornea (Shoham et al., 2008); Nrf2 coordinates upregulation of antioxidant defenses (Espinosa-Diez et al., 2015; Kensler et al., 2007; Osburn et al., 2006; Xu et al., 2014) and the p53 protein coordinates cell cycle arrest, apoptosis, senescence and deoxyribonucleic acid (DNA) repair. Low fluence UV-A irradiation induces Nrf2 regulated antioxidant defences and higher fluence UV-A activates p53 and caspase-3 in corneal endothelial cells (Liu et al., 2016).

UV-B induces homolytic fission of hydrogen peroxide (H2O2) to generate the hydroxyl radical in the cornea and the skin, which can result in damage to macromolecules like DNA and lipids (Shoham et al., 2008). Lipid peroxidation occurs in response to elevated levels of ROS and may result from UV-induced oxidative destruction of cell membranes and the formation of cytotoxic aldehydes in the cornea (Arnal et al., 2011). Irreversible ROS damage results in apoptosis through p53 promotion (Liu et al., 2016). Reactive oxygen species (ROS) are a by-product of oxidative phosphorylation in mitochondria, which can subsequently result in further mitochondrial damage and a further increase in ROS (Brown and DC, 1994; Johns, 1995). The progressively damaged mitochondria become less efficient, lose their functional integrity and release more ROS, thereby exacerbating oxidative damage. This results in progressive accumulation of dysfunctional mitochondria with age. This mitochondrial dysfunction results in the induction of further mtDNA mutations, establishing a ‘vicious cycle’ of diminishing mitochondrial functional activity, subsequent apoptosis and loss of cell viability (Figure 2) (Charles et al., 2005; Green and Reed, 1998; Jiang et al., 2005; Petit et al., 1996; Richter, 1998; Yang et al., 2005). The electrophilic nature of ROS can result in injury to biologic nucleophiles e.g. lipids, proteins and DNA (Cheung et al., 2013). mtDNA is particularly susceptible to oxidative damage and mutation: inefficient DNA repair mechanisms, the high transcription rates of mtDNA, a lack of protection of mtDNA by the histone proteins, as well as the proximity of mtDNA to the inner mitochondrial membrane where it generates the greatest amount of reactive oxygen species (Ballinger et al., 1999; Barja, 2004; Mecocci et al., 1993; Salazar and Van Houten, 1997). Oxidative damage to mtDNA results in defective mtDNA encoded subunits of respiratory chain (creating further oxidative stress) and disturbance in intrinsic apoptotic pathways which collectively results in further cellular damage and tissue dysfunction (Wojcik et al., 2013).

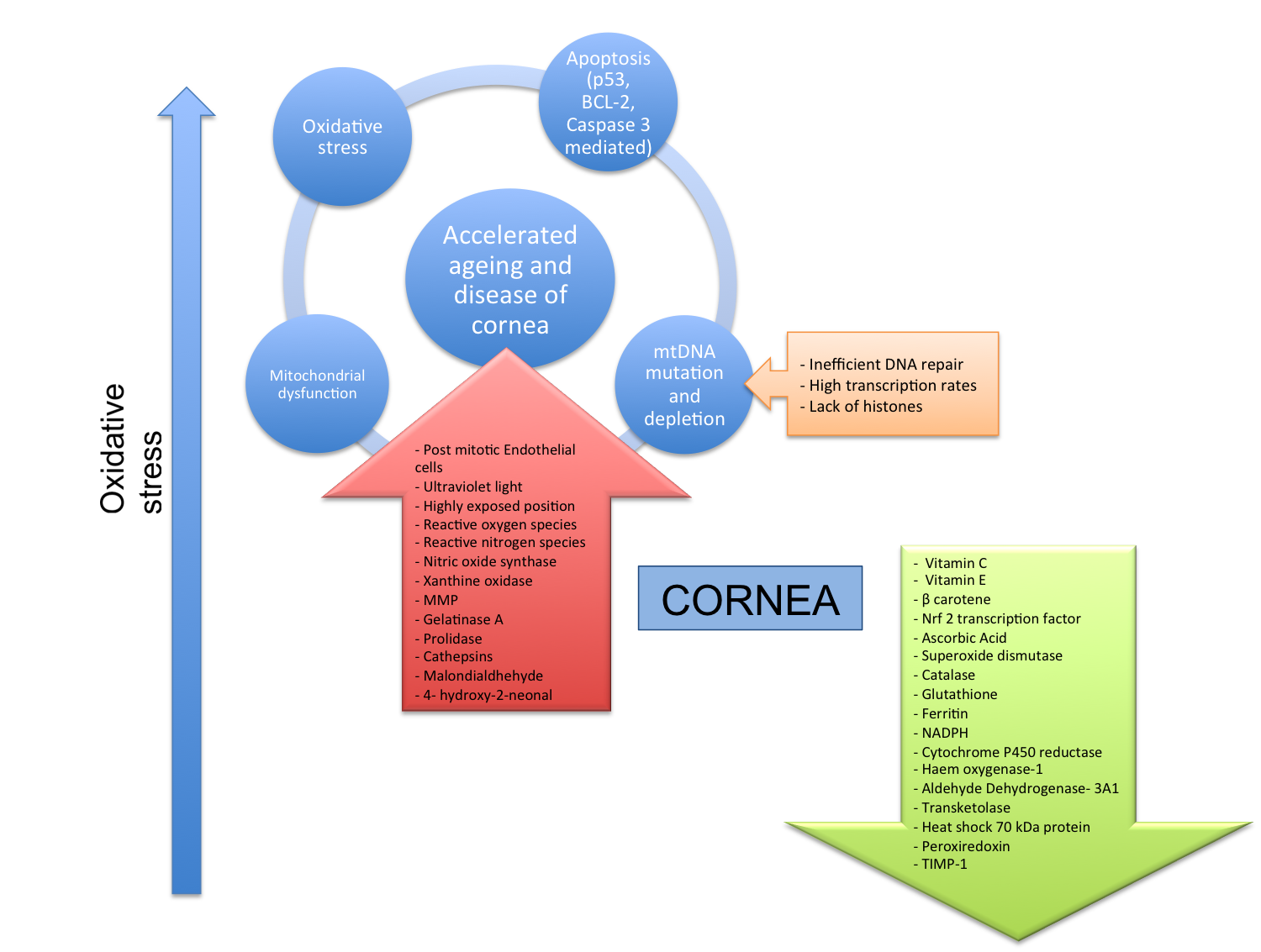
Mutations in mtDNA are usually heteroplasmic, meaning that the same cell can contain varying proportions of mutated and wild-type (normal) mtDNA (as opposed to homoplasmy- the presence of either completely wild type or completely mutant mtDNA)(Johns, 1995). If a mutation is pathogenic, the cell can usually tolerate a specific of this mutant mtDNA variant before the biochemical threshold is exceeded and a defect in the respiratory chain is detected. Typically, this threshold level is >80%, suggesting that most mtDNA mutations are haploinsufficient or recessive (Durham et al., 2007). Consequently, the phenotypic threshold effect whereby the phenotypic manifestation of the genetic defect (mtDNA deletions) occurs when a threshold level of heteroplasmy is exceeded (Rossignol et al., 2003). The corneal endothelium is vulnerable to accumulating mtDNA damage, as despite the post- mitotic nature of endothelial cells, their mitochondrial genome is continually replicating and mtDNA mutations can replicate and increase in number over time. Therefore, the signs and symptoms of corneal endothelial cell dysfunction may not appear until a significant proportion of mitochondria are defective, and this risk is heightened given the natural decline of the endothelial cell count over time (Bourne et al., 1997).

4. **Antioxidants and the cornea**

In the healthy cornea antioxidants such as superoxide dismutase, catalase, ascorbate and glutathione are present (Chwa et al., 2006; Spector et al., 2002; Yamada et al., 1991). Ascorbate (Vitamin C) acts as a reducing agent and reacts rapidly with ROS, such as superoxide anion and hydroxyl radical, thereby protecting the cornea from oxidative stress (Zierhut et al., 2008). Ascorbate acts as a filter from UV light, which is required for the prevention of light induced damage (Ringvold et al., 2003, 1998; Ringvold, 1998). Diurnal species (greater light exposure) have higher concentrations ascorbate in the aqueous humour (Reddy et al., 1998; Reiss et al., 1986) and corneal epithelium (Ringvold et al., 1998) in comparison to nocturnal species. Animal studies analysing reindeer, rabbit and bovine levels of ascorbate show that there are very high concentrations of ascorbate throughout the eye (aqueous humour, corneal epithelium) at almost 10-100x the concentrations found in serum (Ringvold et al., 2003). It has been shown that in cows and humans ascorbate is up to 8 times more concentrated in corneal epithelium and endothelium compared to corneal stroma (Brubaker et al., 2000; Delamere, 1996; Pirie, 1946; Ringvold et al., 2000). The high turnover of the aqueous humour replenishes the loss of ascorbate in the cornea following free radical scavenging (Zierhut et al., 2008).

The three major mammalian superoxide dismutase (SOD) antioxidant enzymes, SOD 1 (CuZnSOD), SOD2 (MnSOD) and extracellular SOD3 (EC-SOD) have been identified in the corneal epithelium, stroma and endothelium (Behndig et al., 1998). The cornea contains unusually large amounts of SOD3, among the highest levels measured in the human body, and close to that of SOD1 (Zierhut et al., 2008). High levels of SOD3 and SOD1 are particularly seen in the corneal epithelium (Zierhut et al., 2008). Catalase in the cornea converts hydrogen peroxide (H2O2) to water and oxygen (Atalla et al., 1987), which protects SOD from hydrogen peroxide mediated inactivation (Cheung et al., 2013). Glutathione peroxidase in the cornea transforms H2O2 to oxygen and water in healthy corneas via the oxidation of glutathione (Cheung et al., 2013; Gondhowiardjo and van Haeringen, 1993). Further antioxidants in the corneal epithelium include nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P450 reductase and haem oxygenase-1. Whereas corneal stromal keratocytes express the anti-oxidants aldehyde dehydrogenase-3A1 and transketolase (Cheung et al., 2013; Shoham et al., 2008). Studies on the rabbit cornea demonstrated that in aged corneas (3.0- 3.5 years) the activities of antioxidant enzymes (SOD, glutathione peroxidase, catalase) were dramatically reduced or even lost, whereas the ROS producing Xanthine oxidase activity was only slightly decreased, suggesting an imbalance is oxidative defence in the cornea with age (Čejková et al., 2004).

**Figure 2- Protective mechanisms and risk factors for mitochondrial dysfunction and oxidative stress in the cornea**

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**5. Corneal endothelial dysfunction in inherited mitochondrial disease**

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of mitochondrial dysfunction caused by mutation of either nuclear DNA or mitochondrial DNA. Many individuals with a mutation of mtDNA display a cluster of clinical features that fall into a discrete clinical syndrome (Chinnery, 2000). A corneal phenotype has been reported in Kearns-Sayre syndrome (KSS; MIM#530000) (Boonstra et al., 2002; Chang et al., 1994; Nakagawa et al., 1995; Ohkoshi et al., 1989) and Pearson syndrome (OMIM#557000) (Kasbekar et al., 2011; Lee et al., 2007).

Kearns-Sayre syndrome (KSS) is a rare neuromuscular disorder usually resulting from a 5 kb deletion in mtDNA (Lestienne and Ponsot, 1988). Clinically KSS is characterised by ocular signs and symptoms, which become apparent before the age of 20 years: presence of ptosis (partial closure of the eyelids due to weakness of elevator palpebrae superioris), progressive paralysis of extraocular muscles (resulting in reduced eye movements) and progressive retinal dysfunction (atypical retinitis pigmentosa). Typically, KSS affects ocular tissues and non-ocular tissues with high metabolic demands due to the inability of the mitochondria to meet these high-energy demands.

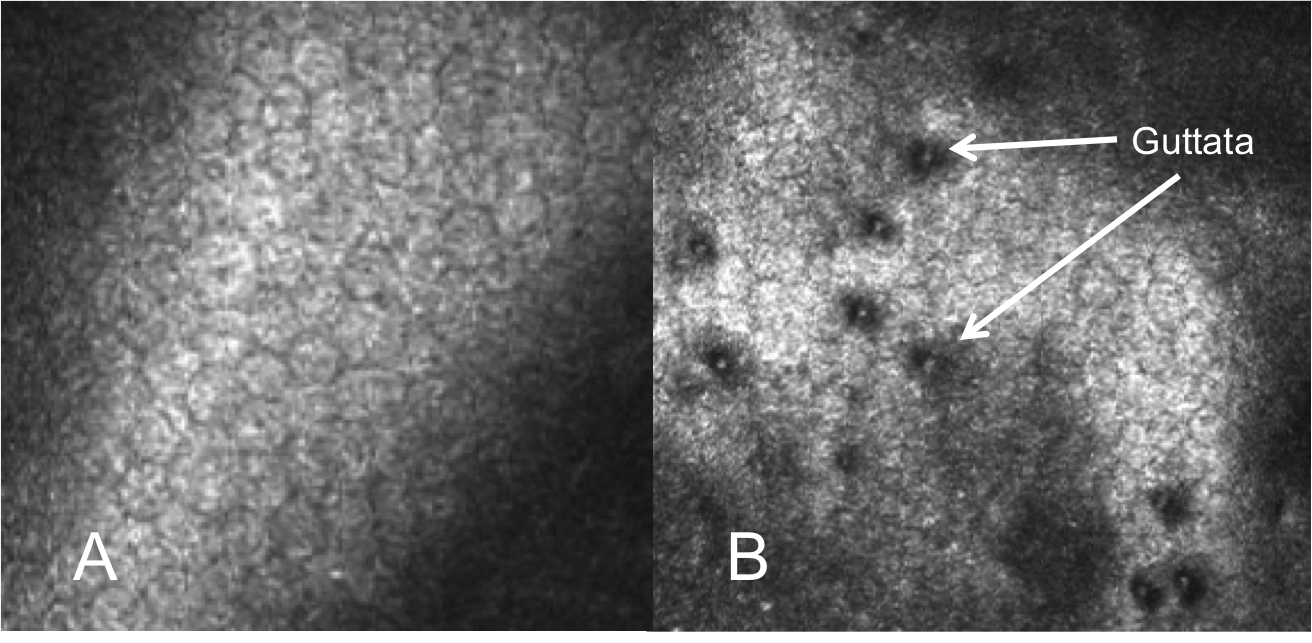
Corneal decompensation due to corneal endothelial dysfunction has been reported in KSS (Boonstra et al., 2002; Chang et al., 1994; Nakagawa et al., 1995; Ohkoshi et al., 1989). Epithelial and stromal oedema of the cornea are seen in KSS (Boonstra et al., 2002), which result in reduction of vision and in advanced cases discomfort and pain. Alterations in corneal endothelial morphology detected using in-vivo specular microscopy have been reported in KSS (Ohkoshi et al., 1989). TEM of the cornea of a patient with KSS showed that the keratocytes were vacuolated in appearance and contained abnormal mitochondria with distorted cristae (Chang et al., 1993). In a second reported case of KSS the corneal epithelium had abnormal mitochondria, and a complete absence of corneal endothelium has also been reported in KSS (Nakagawa et al., 1995). In KSS mtDNA mutation influences endothelial function and consequently corneal transparency. The augmentation of anaerobic glycolysis with lactate accumulation in the corneal endothelium may exacerbate corneal endothelial dysfunction, as the high concentration of lactate has an apoptotic effect, which results in the subsequent polymorphism (variation in CEC size), polymegathism (variation in CEC shape) and the reduction of corneal endothelial cells (Boonstra et al., 2002; Mirabella et al., 2000). These endothelial changes reported in KSS mirror those seen with ageing and in corneal endothelial dystrophies (Chang et al., 1994).

Corneal endothelial dysfunction is therefore a feature in KSS and can lead to corneal decompensation with resultant corneal oedema and visual impairment. Corneal endothelial dysfunction is also seen as part of the ocular phenotype in Pearson syndrome (MIM#557000)(Kasbekar et al., 2011; Lee et al., 2007). Pearson syndrome presents in early infancy with a combination of refractory sideroblastic anaemia, pancytopenia, vacuolization of marrow precursors and exocrine pancreatic dysfunction (Pearson et al., 1979). Pearson syndrome is caused by a mtDNA deletion of 5kB in most cited cases (McShane et al., 1991; Rotig et al., 1989; Rötig et al., 1990) resulting in a multisystem severe deficiency in mitochondrial energy supply (Rötig et al., 1990). Life expectancy is significantly reduced but if prolonged the clinical phenotype can evolve into or demonstrate the clinical and ocular features of KSS (Lee et al., 2007). Corneal findings that have been observed include bilateral stromal haze and complete epithelial oedema with corneal thickening (Kasbekar et al., 2011; Lee et al., 2007). It has been proposed that corneal oedema in patients with Pearson syndrome or evolved Pearson syndrome with KSS results from corneal endothelium pump failure due to the high metabolic demand at this site and its susceptibility to mitochondrial dysfunction (Kasbekar et al., 2011).

**6. Mitochondrial dysfunction and oxidative stress in Fuchs endothelial corneal dystrophy**

Fuchs endothelial corneal dystrophy (FECD) is a progressive, bilateral disease characterised by a gradual loss of corneal endothelial cells (CECs). Loss of CECs impairs the ability of the cornea to maintain hydration and results in a progressive decline in corneal transparency and hence a decline in vision (Bonanno, 2003; Elhalis et al., 2010). FECD is estimated to affect about 4% of the population, mostly in the fourth and fifth decade of life (Wojcik et al., 2013). In FECD there is an accelerated decrease in corneal endothelial cell density (beyond that of normal ageing) and the formation of focal accumulations of collagen and sub-endothelial extracellular matrix at the posterior surface of Descemet's membrane, called guttae (Klintworth, 2009) . Figure 3 demonstrates specular microscopy of normal corneal endothelial cells and those affected by Fuch’s endothelial corneal dystrophy (FECD). The pathogenesis of FECD is not completely understood, but is thought to be multifactorial, with several genetic and environmental factors contributing to its development (Elhalis et al., 2010; Iliff et al., 2012; Jurkunas et al., 2010; Wojcik et al., 2013). Although in the majority of cases FECD is reported as a sporadic disorder, autosomal dominant patterns of inheritance have been reported (Iliff et al., 2012) and a heterozygous intronic trinucleotide repeat expansion (CTG) in the TCF4 gene (602272) has been associated with late-onset FECD (Baratz et al., 2010).

**Figure 3- Confocal microscopy of corneal endothelial cells. (A) Demonstrates normal endothelial cells with a regular hexagonal shape. (B) Fuch’s endothelial corneal dystrophy show a loss of defined hexagonal shape, increased cell size and the formation of guttata (as labelled)**

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The high metabolic activity, exposure to sunlight and the lack of a significant capacity for natural regeneration of CECs make them susceptible to mitochondrial dysfunction and oxidative damage. Corneal tissue from patients affected by FECD show an increased level of ROS and 8-hydroxy-2'-deoxyguanosine (8-OHdG): a marker of the degree of oxidative damage (Jurkunas et al., 2010). FECD patients present with greater levels of oxidative peripheral blood DNA lesions compared with age- and sex-matched controls (although not statistically significant) and significantly lower efficacy of DNA repair in FECD patients than controls (Czarny et al., 2013). DNA lesions (including oxidative damage such as strand breaks, base modifications and abasic sites) block the progression of DNA polymerase so that only undamaged DNA templates can participate in a polymerase chain reaction (PCR). Thus, DNA amplification is inversely proportional to DNA damage: the more lesions encountered on the target DNA, the less the amplification (Santos et al., 2003). A marked decrease in relative nuclear DNA amplification by 95% (p<0.001) has been found in human FECD corneal endothelial cell lines than control cell lines (Halilovic et al., 2016). A reduced number of mitochondria in the endothelium and a decreased activity of cytochrome oxidase in the cornea have also been reported in FECD (Tuberville et al., 1986). An increased level of oxidative DNA damage in areas of corneal guttae was reported, indicating an association between macromolecular damage triggered by oxidative stress and apoptosis of the endothelial cells in FECD (Jurkunas et al., 2010). The majority of DNA damage was located in mtDNA, suggesting that mtDNA is the primary target of oxidative injury, reflected by accumulation of oxidised DNA in the form of 8-OHdG at mitochondrial sites in FECD (Jurkunas et al., 2010). Mitochondrial DNA copy number has also been found to be 80% lower in FECD CECs than normal CECs (p<0.001) (Halilovic et al., 2016).

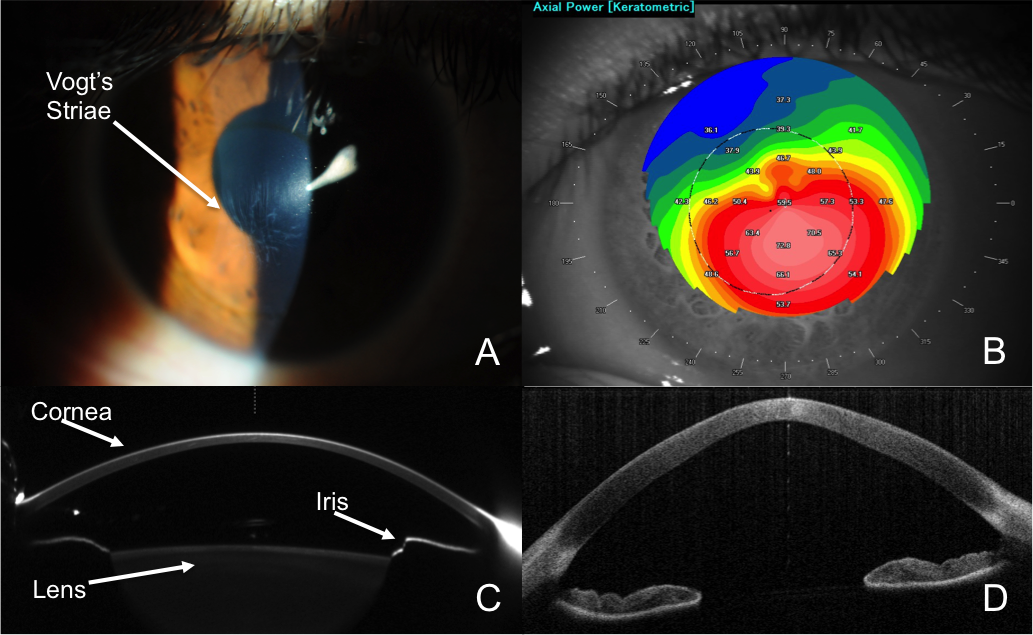
Increased oxidative stress in the FECD cornea contributes to endothelial oxidative DNA damage, morphological modification and CEC apoptosis (Jurkunas et al., 2010). There is a decrease in the antioxidant response element-driven antioxidants in FECD corneal endothelium (Jurkunas et al., 2010). Specifically, there is a down-regulation of transcription factor Nrf2 in FECD endothelium. Nrf-2 mediated antioxidant defence and p53 have been demonstrated to have critical roles in regulating oxidative stress induced apoptosis in FECD (Azizi et al., 2011; Jurkunas et al., 2010). Photo-oxidative injury with formation of ROS has been shown to induce corneal endothelial cell apoptosis in animal models (Ashok and Ali, 1999; Podskochy et al., 2000). UV-A treated human corneal endothelial cells induce Nrf2 antioxidant defence mechanisms which are deficient in FECD and suggest that UV-A may impact on the abnormal CEC physiology in FECD (Liu et al., 2016). Sulfolane treatment of FECD (an agonist of Nrf2) and normal ex vivo corneas and human corneal endothelial cell lines, resulted in a cytoprotective effect with decreases CEC apoptosis due to an over-expression of antioxidant molecules and reduction in intracellular ROS production (Ziaei et al., 2013).

The normal physiology of the corneal antioxidants are also affected in FECD (Buddi et al., 2002; Elhalis et al., 2010; Gottsch et al., 2003; Jurkunas et al., 2008, 2010; Nordberg and Arnér, 2001). Proteomic analyses in FECD cells demonstrated a downregulation of glutathione S-transferase, aldehyde dehydrogenase 3 (ALDH3A1), ferritin, heme oxygenase 1 (HO-1), heat shock 70-kDa protein and peroxiredoxins (Buddi et al., 2002; Gottsch et al., 2003; Jurkunas et al., 2008, 2010a). Peroxiredoxins and heat shock 70-kDa may play an important role in FECD as both are involved in the process of apoptosis (Gottsch et al., 2003; Jolly and Morimoto, 2000). In the endothelium of FECD corneas a reduced level of SOD2 in the mitochondrial matrix, metallothionein 3 (MT3), and thioredoxin reductase 1 (TXNRD1) (Jurkunas et al., 2010) have been reported (Nordberg and Arnér, 2001) These are important in the regeneration of various low molecular weight antioxidants, including vitamin E, vitamin C, lipoic acid and superoxide. The downregulation of antioxidant enzymes in the cornea and its endothelium can lead to perturbation of tissue homeostasis and activation of apoptotic pathways in FECD (Gottsch et al., 2003).

**7. Mitochondrial dysfunction and oxidative stress in keratoconus**

Keratoconus is the leading cause of corneal transplantation in young people accounting for 25% of all transplants. Keratoconus affects around 1 in 2000 people in the United Kingdom. It is a lifelong condition, and is a significant health burden in work-age adults (Rabinowitz, 1998). The corneal thinning and protrusion due to keratoconus commences in teenage years with variable progression into mid-life, at which point it normally stabilises (see Figure 4)(Rabinowitz, 1998). Clinically, the severity of keratoconus ranges from myopia and astigmatism to severe progressive conical protrusion, scarring, or blindness. Most patients require contact lenses of increasing complexity and 1/5th of patients require corneal transplants; keratoconus is the second commonest indication for corneal transplantation in the UK (Keenan et al., 2012).

**Figure 4- Findings of Keratoconus. (A) Colour slit-lamp photo of a keratoconic (conical shaped) cornea showing subepithelial scarring and Vogt’s striae (B) Keratometric map (corneal topography) of keratoconic changes in cornea showing inferotemporal cone with increased corneal power (red area) (C) Anterior segment Optical Coherence Tomography (OCT) of normal cornea showing the smooth profile of the cornea. (D) Anterior segment OCT of keratoconic cornea showing the altered corneal profile with a conical appearance.**



Several studies have analysed mtDNA changes in peripheral blood leucocytes isolated from keratoconic patients from specific ethnic groups (K K Abu-Amero et al., 2014; Khaled K. Abu-Amero et al., 2014; Hao et al., 2015; Tanwar et al., 2010). Sanger sequencing of the mtDNA genome from peripheral blood leucocytes in 26 keratoconic patients from the Saudi population detected potentially pathogenic nonsynonymous mtDNA mutations in 38.5% (10/26 cases); absent from ethnically matched controls. One nonsynonymous mtDNA sequence change was heteroplasmic, whereas all the remaining 9 were homoplasmic (Khaled K. Abu-Amero et al., 2014). Sequencing of the mitochondrial complex I genes (ND1, 2, 3, 4, 4L, 5, and 6) in 20 keratoconus cases from India detected 18 nonsynonymous and two novel frame shift mutations reported as pathogenic (Pathak et al., 2011). The authors proposed that these complex 1 mutations might account for increased ROS and the development of keratoconus (Pathak et al., 2011). Interestingly complex 1 gene mutations are also observed in other ocular conditions including Leber’s Hereditary optic neuropathy (Carelli et al., 2004), glaucoma (Sundaresan et al., 2014) and oncocytomas (Zimmermann et al., 2011).

A significant increase in the mean mtDNA content (the number of mitochondria in a cell and the amount of mtDNA) of peripheral blood leucocytes was reported patients with keratoconus patients compared to controls in a Saudi Arabian population (K K Abu-Amero et al., 2014). Similarly, mtDNA copy number was significantly higher in patients with keratoconus compared to controls (K K Abu-Amero et al., 2014). However, a significant decrease in mtDNA copy number of keratoconic patients compared to controls was reported in the Chinese population and further work is required to understand these differences (Hao et al., 2015). Using mitochondrial haplogrouping in the Saudi population haplogroups H and R were significantly overrepresented in keratoconic patients compared to controls. Conversely in a cohort of Chinese keratoconic patients (n=210) no haplogroup association was detected (Hao et al., 2015). The authors suggested the different matrilineal genetic backgrounds of these two populations (Hao et al., 2015) with haplogroup H originating in the Near and Middle East explained these findings (Roostalu et al., 2007).

Studies on the corneal tissue obtained from keratoconic patients undergoing corneal transplantation have detected mtDNA changes (Atilano et al., 2009, 2005; Brown et al., 2004; Buddi et al., 2002; Kenney et al., 2005). Keratoconic patients (n=35) have increased numbers of mtDNA deletions on PCR screening compared to control tissue (Atilano et al., 2005). Reduced immunohistochemical expression of cytochrome c oxidase (complex IV) subunit 1(CO-I) in the basal epithelial cells in areas of corneal thinning (active disease) has been reported (Atilano et al., 2005). Immunohistochemical staining of 8-OH-dG was present in all layers of epithelial cells and stroma in keratoconic corneas but only in superficial layers of normal corneal epithelium; providing further evidence of oxidative DNA damage in keratoconus (Atilano et al., 2009; Brown et al., 2004; Buddi et al., 2002; Kenney et al., 2005). Accumulation of ROS/RNS can impact cells by increasing degradative enzyme activities and decreasing collagen synthesis, which is particularly relevant in conditions such as keratoconus (Brenneisen et al., 1997; Brown et al., 2004; Siwik et al., 2001). Keratoconic corneal fibroblasts show an increased production and increased susceptibility of ROS and RNS when compared with normal corneal fibroblasts (Chwa et al., 2006; Pathak et al., 2011) and are more susceptible to stressful challenges (low pH, and hydrogen peroxide) than normal corneal stromal cells (Chwa et al., 2008)*.*

Chronic keratocyte apoptosis, particularly of anterior stromal keratocytes, can lead to stromal thinning in keratoconus (Kim et al., 1999; Thalasselis, 2005). Vesicular degeneration and swelling of mitochondria was observed in the cytoplasm of the epithelial cells of 20 keratoconic corneas with electron microscopy (Aktekin et al., 1998). Histological features and the atypical expression of a variety of apoptosis related molecules have been demonstrated in keratoconus; including DNA fragmentation, cell shrinkage, budding of apoptotic bodies and chromatin condensation and fragmentation (Becker et al., 1995; Fabre et al., 1991; Lee et al., 2009; Rabinowitz et al., 2005; Zhou et al., 1996).

Reactive nitrogen species (RNS) have also been implicated in the pathogenesis of keratoconus and studies have shown accumulation of nitrotyrosine, a marker for the formation of peroxynitrite, in keratoconic corneas compared with normal corneal tissue (Buddi et al., 2002). Nitric oxide synthase converts L-arginine into nitric oxide (NO), another RNS, and elevated levels of endothelial nitric oxide synthase (eNOS) at the site of Bowman’s layer breaks was observed in keratoconus, indicating an increased production of nitric oxide in these corneal regions (Becquet et al., 1997; Jurkunas et al., 2010; Sennlaub et al., 1999). In conditions of oxidative stress, NO is synthesised by corneal keratocytes (Sennlaub et al., 1999), and NO is present in significantly higher levels in keratoconic patients (Arnal et al., 2011). The peroxynitrites, NO and ROS result in cytotoxic effects damaging DNA, degrading tissue inhibitors of matrix metalloproteinases (TIMPs) and activating matrix metalloproteinase -2 (MMP-2) (Brown et al., 2004). A 1.8 fold reduction in levels of TIMP-1 has been seen in keratoconic corneas (Kenney et al., 2005). TIMP-1 plays a role in inhibition of apoptosis in a variety of cell types, hence a reduction is associated with fragmentation of the epithelium and stromal thinning of keratoconic corneas (Nita and Grzybowski, 2016; Smith and Easty, 2000). Keratoconic corneas exhibit increased activity of gelatinase A (Collier, 2001; Smith et al., 1995). MMP and gelatinase A are proteolytic enzymes that catalyse the digestion of extracellular matrix components e.g. collagen IV, collagen V, fibronectin and laminin (Brown et al., 1993; Nita et al., 2014; Zhou et al., 1998). Prolidase is a manganese dependent matrix metalloproteinase also involved in collagen degradation and matrix remodelling (Miltyk et al., 2008; Surazynski et al., 2005). There are significantly lower levels of prolidase in the serum of patients with keratoconus than controls (Akal et al., 2015; Kılıç et al., 2016). Increased levels of cathepsins B, G and V/L2 and other lysosomal enzymes are found in keratoconic corneas (Brookes et al., 2003; Kenney et al., 2005; Whitelock et al., 1997; Zhou et al., 1998); cathepsins can elevate hydrogen peroxide levels, thus increasing oxidative stress and apoptosis (Cirman et al., 2004; Guicciardi et al., 2000; Ishisaka et al., 1999; Zhao et al., 2003).

Altered antioxidant levels have also been reported in the keratoconic cornea (Arnal et al., 2011; Buddi et al., 2002; Chwa et al., 2006; Gondhowiardjo and van Haeringen, 1993; Kenney et al., 2005; Pappa et al., 2003) A 2.2 fold increase of protective catalase messenger ribonucleic acid (mRNA) levels (p<0.01) (Kenney et al., 2005) and a significant reduction in levels of the antioxidant glutathione (Arnal et al., 2011) have been found in keratoconic corneas compared to normal matched controls. Keratoconic corneas have been reported to have an elevated level of reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are produced by lipid peroxidation and can damage cellular biomolecules (Arnal et al., 2011; Buddi et al., 2002; Uchida, 2000). These aldehydes can also cause changes in the membranes of lysosomes, which in turnrelease proteolytic enzymes (Kalra et al., 1994). Keratoconic corneas exhibit reduced levels of aldehyde dehydrogenase 3 (ALDH3), which detoxifies reactive aldehydes produced by UV- induced lipid peroxidation (Chwa et al., 2006; Gondhowiardjo and van Haeringen, 1993; Pappa et al., 2003) .

Keratoconic intact corneas and keratoconic corneal fibroblasts placed under conditions of stress (low pH, exposure to H2O2) both showed increased catalase activity, ROS production and apoptosis(Chwa et al., 2008). Protein levels of extracellular superoxide dismutase (SOD3) in keratoconus are significantly reduced, to about half of the levels in the normal central cornea; the levels of SOD1 and 2 are unaltered (Behndig et al., 2001, 1998). Interestingly the mRNA levels of SOD3 were similar in keratoconic corneas and control tissue and this difference between SOD3 mRNA and protein levels may be due to post translational processes e.g. enzyme turnover rates, genetic polymorphisms, response to cytokines, oxidative stress or trauma (Kenney et al., 2005; Zierhut et al., 2008). Mutational analysis of SOD1 in keratoconus has resulted in conflicting results (De Bonis et al., 2011; Saee-Rad et al., 2011; Udar et al., 2009). A heterozygous 7 base deletion in intron 2 of SOD 1 has been reported in two keratoconus families (Udar et al., 2006). However a role for SOD1 mutation in keratoconus has not yet been firmly established (De Bonis et al., 2011; Saee-Rad et al., 2011; Udar et al., 2009).

**8. Anti-oxidant and mitochondrial therapies for corneal disease**

Corneal epithelial defects are commonly treated using agents known for their antioxidant properties e.g. ascorbate (Vitamin C)(Boyd and Campbell, 1950; Gross, 2000; Saika et al., 1993). Other antioxidant agents such as Trolox® (a cell permeable, water soluble derivative of vitamin E) (Hallberg et al., 1996), vitamin E (Bilgihan et al., 2003) and superoxide dismutase derivatives e.g. an acylated SOD derivative (Ando et al., 1990) and lecithin bound superoxide dismutase (Shimmura et al., 2003) can accelerate epithelial healing. In the corneal stroma antioxidant agents and superoxide dismutase derivatives in rabbit wound healing models have successfully treated deep corneal wounds involving the stroma (Ando et al., 1990) and this was replicated in human studies (Alio et al., 1995; Vetrugno et al., 2001). Coenzyme Q10 containing eye drops in rabbits reduce corneal damage after UV-B exposure *in vivo* and *in vitro* by preserving mitochondrial function and maintaining oxygen consumption at control levels (Mencucci et al., 2014). The increasing evidence of mitochondrial abnormalities in corneal disease is catalysing the development of novel therapeutic agents targeting mitochondrial function to treat corneal disease (Halilovic et al., 2016). Stealth Biotherapeutics have developed a topical eye formulation (Elamipretide®), which is in a phase 2 clinical trial (ReVEAL) for the treatment of Fuchs endothelial corneal dystrophy. Elamipretide® targets the inner mitochondrial membrane, preserving mitochondrial energetics and restoring normal energy production in mitochondria (Azevedo, 2016). Elamipretide® contains novel, water soluble peptide, named MTP-131 or SS-31, which has a highly favourable structure for membrane penetration, targeting mitochondria in a potential independent manner (Chen et al., 2011).

9. **Conclusion**

This review demonstrates the increasing evidence of the role oxidative stress and subsequent mitochondrial dysfunction in the pathogenesis of both inherited and acquired corneal pathologies. The unique physiology of the cornea predisposes this structure to oxidative damage, and there is interplay between inherited and acquired mitochondrial dysfunction, oxidative damage and a number of corneal diseases. The imbalance of ROS and antioxidants can then result in mtDNA mutations which can play a significant role in cellular dysfunction, through inefficient energy production by mitochondria and cellular apoptotic pathways. The progressive accumulation of mtDNA mutations can result in high levels of heteroplasmy beyond the phenotypic threshold hence resulting in disease. The corneal endothelium is vulnerable to accumulating mtDNA damage, as despite the post- mitotic nature of endothelial cells, their mitochondrial genome is continually replicating and mtDNA mutations can replicate and increase in number over time. By targeting mitochondrial dysfunction in corneal disease emerging treatments may prevent or reduce visual loss.

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(Figure 3)

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