ROS

REVIEW ARTICLE

Oxidative Stress in the Pathogenesis of Corneal Endothelial Dystrophies and Other Corneal Diseases

Sanjukta Guha[1,3], Sunita Chaurasia[2], and Sanhita Roy[1,2]

[1]Brien Holden Eye Research Centre, LVPEI, Hyderabad, India; [2]Tej Kohli Cornea Institute, LVPEI, Hyderabad, India; [3]Manipal Academy of Higher Education, Manipal, India

Correspondence: sanhita@lvpei.org (S.R.)

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ABSTRACT | Cornea being constantly exposed to sunlight and atmospheric oxygen is readily prone to oxidative damages. The functional antioxidant signaling helps maintain the levels of reactive oxygen species in the cells and keeps the cornea healthy. Corneal endothelial dystrophies include a group of corneal diseases that are marked by progressive degeneration of corneal endothelium leading to loss of vision. The increased level of oxidative stress in several corneal endothelial dystrophies indicates that there might be disruption in proper functioning of these signaling pathways. Various strategies to improve antioxidant signaling pathways may help develop novel clinical interventions to combat degeneration of endothelial cells that leads to vision loss. This review gives an overview of oxidative stress in the pathogenesis of corneal diseases.

KEYWORDS | Congenital hereditary endothelial dystrophy; Corneal endothelial dystrophy; Nrf2; Oxidative stress; Reactive oxygen species; Keratoconus; Fuchs' endothelial corneal dystrophy; Posterior polymorphous corneal dystrophy; Granular corneal dystrophy type 2; Schnyder corneal dystrophy

ABBREVIATIONS | ALD, aldehyde dehydrogenase; CHED, congenital hereditary endothelial dystrophy; FECD, Fuchs' endothelial corneal dystrophy; GCD2, granular corneal dystrophy type 2; HO-1, heme oxygenase 1; MDA, malondialdehyde; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PPCD, posterior polymorphous corneal dystrophy; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCD, Schnyder corneal dystrophy; SOD, superoxide dismutase; UV, ultraviolet

CONTENTS

1. INTRODUCTION

In recent years, oxidative stress has not only been associated with several conditions such as aging, infertility, cancer, and neurodegenerative disorders, but also been marked as a target for therapeutic interventions for these diseases. Oxidative stress has also played a pivotal role in numerous ocular diseases. Some of these diseases include cataract [1], open-angle glaucoma [2], age-related macular degeneration [3], uveitis [4, 5], and retinopathy of prematurity [6]. The cornea, iris, and lens constitute the anterior part of the eye and are constantly exposed to the outside environment comprising of radiation, industrial smoke, vapors, and chemical gases. This leads to the initiation of oxidative stress in the cornea which changes its optical properties leading to loss of vision. The cornea is the transparent front portion of the eye which consists of numerous sensory nerves, originating from the ophthalmic branch of the trigeminal nerve [7]. The cornea is transparent, avascular, and has numerous immature resident immune cells. The transparency is due to lack of blood vessels as well as the uniform collagen fibrils which are arranged in a regular lattice so that scattered light is destroyed by the mutual interference. Malfunctions in any of these components can lead to the loss of transparency of the cornea that is crucial for normal vision.

The cornea has five layers, namely, corneal epithelium, Bowman's layer, corneal stroma, Descemet's membrane, and corneal endothelium. The corneal endothelium is the innermost layer of the cornea which consists of a single layer of hexagonal cells on the inner surface of the cornea [8]. The endothelium oversees the fluid and solute transport across the posterior surface of the cornea and retains the cornea in a hydrated state vital for optical transparency. The endothelium is obtained from the neural crest and is attached to the rest of cornea through the Descemet's membrane, an acellular layer mostly composed of collagen. Human corneal endothelium has around 3,500 cells/mm^2 which are arrested in G1 phase and do not proliferate in vivo [9]. These cells work by a Na^+/K^+-ATPase ion pump to remove water from the stroma and deposit it in the aqueous humor. Fluid can disrupt the highly organized lamellar collagen matrix and lead to a loss of corneal clarity [10]. Corneal dystrophies are a cluster of genetically determined corneal diseases that affect vision in varying ways. Of about twenty different types of corneal dystrophies, endothelial dystrophies are the most common ones. Corneal endothelial dystrophies are characterized by a defect in the active fluid transport by the corneal endothelium, causing corneal edema which leads to loss of corneal transparency and reduced visual acuity. Depending on the layer of the cornea involved, they can be divided into three groups clinically. Abnormalities associated with corneal epithelium, Bowman's membrane, and anterior stroma are grouped under superficial corneal dystrophies whereas stromal corneal dystrophies are associated with stroma. Diseases associated with Descemet's membrane and corneal endothelium are classified as corneal endothelial dystrophies.

The most common form of corneal endothelial dystrophy is Fuchs' endothelial corneal dystrophy (FECD), a slowly progressing degeneration of the corneal endothelium that develops small excrescences called "guttata" on Descemet's membrane. FECD is bilaterally symmetrical and is symptomatic in third or fourth decade of life. Fuchs' dystrophy is also characterized by endothelial cell death with hypertrophy and polymorphism of neighboring cells. Congenital hereditary endothelial dystrophy (CHED), second most common type of corneal endothelial dystrophy, is an autosomal recessive rare condition triggered by homozygous or heterozygous mutations in *SLC4A11* gene, a Na^+/OH^- transporter [11]. CHED is generally categorized by diffused bilateral clouding in both the corneas of an infant. The morphology of the endothelium is highly altered with enormous deposition of collagen secretion, due to which the cornea appears opaque. Posterior polymorphous corneal dystrophy (PPCD) is caused by a heterozygous mutation in the promoter region of *OVOL2* gene [12]. Patients with PPCD usually present with metaplasia and overgrowth of corneal endothelial cells. The inception of the disease is generally in the second or third decade of life. The

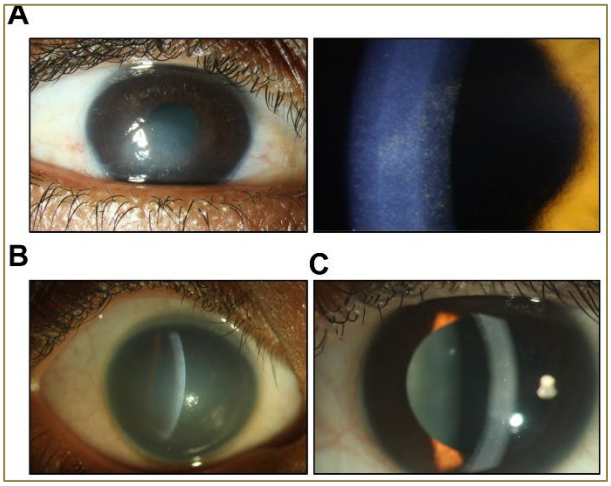

FIGURE 1. Clinical slit lamp images of different types of corneal endothelial dystrophy. Representative images from FECD (A), CHED (B), and PPCD (C) patient.

clinical appearances for different types of corneal endothelial dystrophy are shown in **Figure 1**.

2. OXIDATIVE STRESS AND ANTIOXIDANT SIGNALING

Oxidative stress results from elevated levels of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) that lead to the damage of lipids, proteins, and nucleic acids. Oxidative stress is a causative factor for numerous pathological conditions like neurological disorders, cancer, hypertension, brain ischemia/hypo-perfusion, atherosclerosis, diabetes, acute respiratory distress syndrome, idiopathic pulmonary, asthma, and chronic obstructive pulmonary disease [13–19]. Although high levels of ROS are deleterious, low or moderate amounts of ROS are required to maintain normal physiological functions such as host defense, signal transduction, cell proliferation, and gene expression [20]. ROS are produced under normal conditions due to the partial reduction of molecular oxygen. Superoxide radicals are manufactured mainly by the mitochondria during the oxidative phosphorylation pathway [21]. Any damages in the mitochondria lead to excess production of oxygen radicals [22]. ROS include superoxide anion ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals. On the other hand, nitric oxide radical ($NO^{\bullet}$), peroxynitrite ($ONOO^{-}$), and nitrogen dioxide radical ($NO_2^{\bullet}$) collectively constitute RNS [23]. Under physiological conditions, a delicate redox balance exists between generation and removal of ROS. Eukaryotes have a specialized antioxidant defense mechanism to counteract the ROS, which includes several enzymatic and non-enzymatic molecules. However, the antioxidant defense pathway can be overwhelmed during severe pathological conditions. A key player in the antioxidant defense pathway is leucine-zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) encoded by the gene NFE2L2 and belongs to the basic leucine zipper subset of the Cap 'n' Collar family [24]. Under basal conditions, Kelch-like ECH associated protein1 or Keap1 is tightly bound to Nrf2 and acts as an adaptor for the Cul-3 based E3 ubiquitin ligase complex leading to the degradation of Nrf2. In any events of stress, Nrf2 is activated by modifications of cysteine residues and promptly transported to the nucleus where it binds with other transcription factor like c-Jun and Maf proteins. This complex then binds to the antioxidant response elements in the promoter region which in turn activates transcription of several antioxidant genes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), ferritin, and glutathione reductase (GR) that help detoxify ROS [24] (**Figure 2**). Excessive ROS generation or dysfunctions of antioxidant signaling pathways can results in oxidative stress.

The principal function of the cornea is to refract light to the lens and the retina [25]. Constant exposure of the cornea to ultraviolent rays leads to the production of ROS in cornea. Excessive ROS, if not encountered by antioxidants, makes the cornea vulnerable to oxidative stress [26]. The electromagnetic spectrum of ultraviolet (UV) radiation can be divided into UV-A, UV-B, and UV-C, of which UV-A is absorbed primarily by the lens and UV-B by the cornea. This implies that UV-B contributes significantly to the molecular changes taking place in the cornea [27, 28]. For optimum cell growth, proliferation, differentiation, and apoptosis, ROS and RNS are vital, but excessive production and accumulation of these species are harmful for the cellular organization [20]. Oxidative modifications in lipids, protein, sugars, carbohydrates, and nucleic acids can be attributed to the presence of excessive ROS and RNS which leads

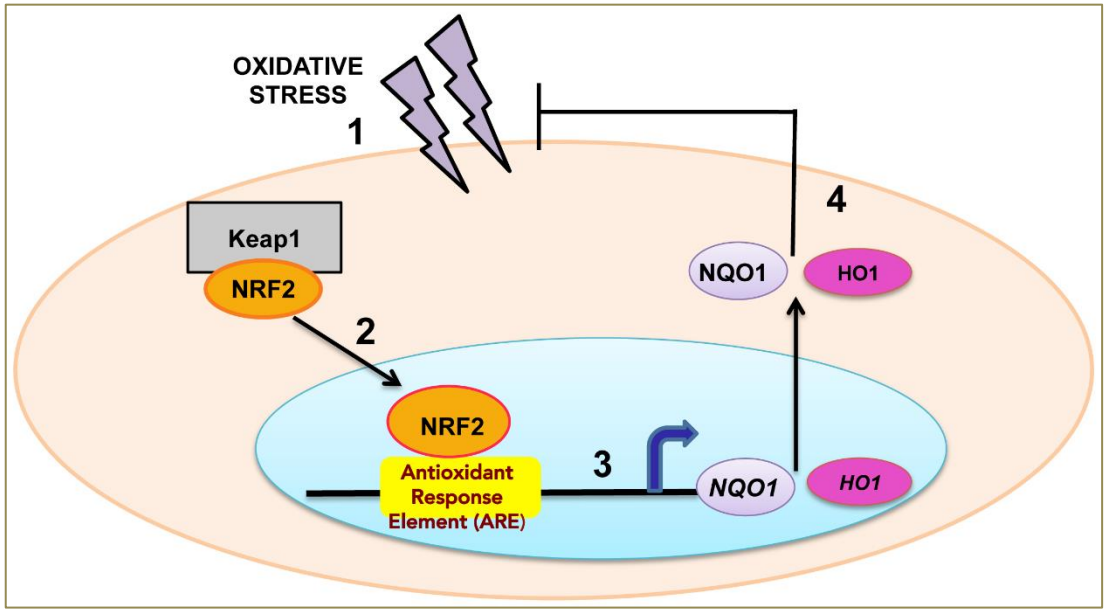

FIGURE 2. Nrf2-mediated antioxidant signaling in response to oxidative stress. The antioxidant signaling in response to oxidative stress in cells is regulated by Nrf2 as detailed in the figure: (1) Cells upon sensing oxidative stress phosphorylates Nrf2 which is normally sequestered in the cytoplasm by Keap1; (2) Nrf2 then translocates into the nucleus and binds to the antioxidant response element of the antioxidant genes; (3) The transcription of antioxidant genes like NQO1 and HO-1 gets upregulated by Nrf2; and (4) The antioxidant genes expressed then inhibit the oxidative stress and maintain the redox balance of cells.

to tissue damage and ultimately cell death. Under various conditions, when cells lose its ability to combat excessive free radicals, corneal diseases occur. The common element found in FECD, CHED, and keratoconus is the presence of elevated levels of ROS, implying that oxidative stress is involved in the progression and development of these diseases.

3. OXIDATIVE STRESS IN CORNEAL ENDOTHELIAL DYSTROPHY

Corneal endothelial dystrophy is a term used for a group of diseases that are characterized by progressive degeneration of corneal endothelium due to both genetic disposition and environmental factors.

3.1. Oxidative Stress in FECD

Ernst Fuchs, an Austrian ophthalmologist in 1910 described FECD as a "dystrophia epithelialis corneae". FECD is a form of corneal dystrophy that usually develops during the third or fourth decade of life. FECD is a slowly progressive disorder which affects approximately 4% of the population in the United States of America, and penetrating keratoplasty (PK) and Descement's stripping endothelial keratoplasty (DSEK) are the only modes of treatment at present [29]. In Fuchs dystrophy, endothelial cells die off and the cornea fills with water, which results in the degeneration of the corneal endothelium and increased accumulation of extracellular excrescences, called guttae. The number of corneal endothelial cells becomes dangerously low and the cornea slowly swells up and ultimately leads to loss of vision [30].

FECD is a multifactorial disease in which genetics and environmental factors play an important role in its development and progression [31]. Mutations in the *COL8A2* gene, encoding α2 subtype of collagen VIII is suggested to be associated with the familial form of FECD [32]. Apart from the *COL8A2* muta-

tions, a heterozygous mutation in *SLC4A11* gene is thought to be involved in the development of the disorder [33]. Depletion of *SLC4A11* has been shown to be associated with increased apoptosis in these cells. *TCF8*, encoding a zinc finger transcription factor, is also considered as a suitable candidate gene for the disease [34]. Elhalis et al. did a family-based study in which they identified two FECD susceptible loci—13pTel-13q12.13 and 18q21.2-q21.32 [31]. Potential linkage regions on chromosomes 1, 7, 15, 17, and X were identified by a genome wide linkage analysis study done on patients with familial FECD [35]. Even with the identification of several genes and chromosome loci associated with the disease, the exact role of the regions in the pathogenesis of FECD still remains unclear.

Several studies show a strong relation between FECD and oxidative stress. Elevated amounts of ROS and RNS products are found accumulated in FECD corneas compared to normal corneas. Interestingly, FECD corneas also show an increased level of nitric oxide synthase in comparison to normal tissues [36]. Also, Gottsch and his colleagues showed that glutathione *S*-transferase (GST), ALDH3A1, and ferritin are down-regulated in FECD cells [37]. Removal of hydrogen peroxide from the cells and inhibition of ROS-induced apoptosis are mediated by peroxiredoxins, and FECD corneas express a low level of peroxiredoxins [38]. FECD endothelial cells exhibit low levels of superoxide dismutase 2 (SOD2) in the mitochondrial matrix along with metallothionein 3 (MT3) and thioredoxin reductase 1 [39]. Thioredoxin reductase 1 catalyzes the regeneration of several low molecular weight antioxidants like vitamin C, lipoic acid, and vitamin E [40]. 8-Hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage was found to be up-regulated in FECD endothelium. DNA damage was mostly found in the mitochondrial DNA, which suggests that oxidative stress primarily targets the mitochondria in turn leading to reduced activity of cytochrome c oxidase, the major respiratory chain enzyme in FECD corneas. Jurkunas et al. have shown loss of mitochondrial membrane potential in ex vivo mouse corneas in the presence of oxidative stress. Several reports suggest a strong association between mitochondrial DNA damage and aging [41]. Halilovic et al. have shown that mechanism of endothelial cell death in FECD was due to increased generation of superoxide in mitochondria and loss of adenosine triphosphate (ATP) [42]. The same study also showed that oxidative DNA damage causes degeneration of endothelial cells in FECD.

3.2. Oxidative Stress in CHED

CHED was first described in 1960 by Edward Maumenee [43], who reported a series of cases of varying corneal clouding that would be congenital and principally stationary. It is a rare subtype of posterior corneal dystrophy characterized by a diffuse ground-glass appearance of the corneas and marked corneal thickening from birth, and blurred vision. In the healthy cornea, the endothelium exists as a monolayer of polygonal cells, which serves as a fluid "pump" to maintain the hydration of the stroma dehydrated and sustains corneal clarity [44, 45]. Although the incidence of CHED is quite low in western world, it is more common in places with higher consanguinity. In reviews from the Middle East and India, CHED accounted for 21% of all pediatric keratoplasty [46]. CHED leads to progressive opacity of the cornea and gradual vision loss and has been associated with mutations in the *SLC4A11* gene [11, 47]. SLC4A11 is an anion transporter present as dimer in the plasma membrane with a molecular weight of 100 KDa. It belongs to the super family of solute carrier 4 (SLC4). Although it was earlier thought to be a borate transporter [48], it recently has been shown to display Na^+ coupled OH^- transport in bovine corneal endothelial cells [49]. Several reports indicate that oxidative stress plays a significant role in the degeneration of the corneal endothelium. The depletion of *SLC4A11* resulted in increased apoptosis of human corneal endothelial cells and hence loss of functional *SLC4A11* is believed to be a causative factor of corneal endothelial cell death [50]. Our laboratory has earlier reported that cells expressing mutant SLC4A11 are more prone to oxidative damages than cells expressing the wild-type protein. The mutant SLC4A11-expressing cells show increased levels of ROS, mitochondrial dysfunction, increased apoptosis and reduced expression of antioxidant genes like *HO-1* and *NQO1* along with Nrf2 [51]. Recently, we have also shown the presence of oxidative stress in corneal tissue sections obtained from CHED patients undergoing corneal transplantation (**Figure 3**) [52]. Increased nitrotyrosine staining was observed in diseased corneas (**Figure 3a**) compared to the cadaveric control corneas (**Figure 3b**). It was also found that depletion of *SLC4A11* hinders Nrf2-mediated antiox-

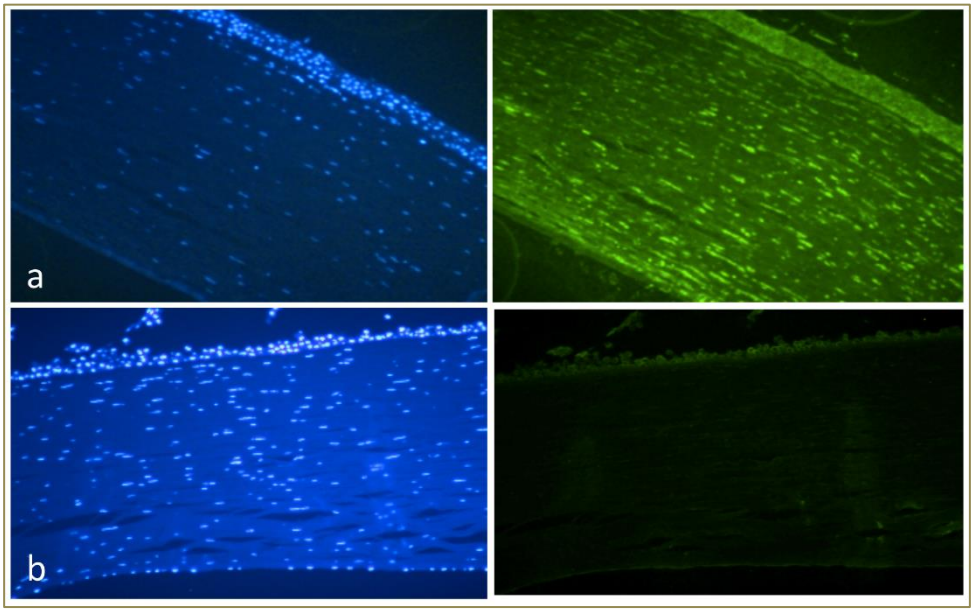

FIGURE 3. Evidence of oxidative stress in CHED tissue specimens. Sections of CHED cornea (a) and cadaveric cornea (b) were immunostained with an anti-nitrotyrosine antibody to detect the presence of oxidative stress and were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Sections were imaged using a fluorescence microscope. This figure was reproduced from ref. 52.

idant signaling and increases ROS in human corneal endothelial cells in the presence of oxidative stress.

3.3. Oxidative Stress in PPCD

PPCD was first described by Koeppe in1916 as "keratitis bullosa interna", due to the presence of congenital pits on the posterior corneal surface [53] and the pathologic descriptions found were abnormalities in Descemet's membrane consisting of fusiform excrescences. PPCD is a rare, bilateral, autosomal dominant and non-progressive disorder that affects the corneal endothelium and Descemet's membrane. The phase-contrast microscopic images done by Henriquez et al. on the corneal button taken from PPCD patients, revealed the irregularly thickened epithelium with an edematous basal cell layer [54]. The Bowman's layer showed focal disruption with penetration of epithelial cells into the superficial stroma. The posterior surface of all corneas had areas of attenuation or discontinuity of the endothelial cell layer. Transmission electron microscopy (TEM) studies show thin, attenuated endothelial cells with disorganized organelles, phagosomal inclusions, and destruction of the cell membrane. The epithelial-like cells were often multi-layered with numerous microvillous projections on the surface facing the anterior chamber. Desmosomal attachments and kerato-fibrils were particularly prominent [54]. In general, PPCD can be characterized by vesicles, bands, and polymorphous opacities in the Descemet's membrane and corneal endothelium.

PPCD patients are often asymptomatic until middle age and visual impairment occurs due to corneal edema in very few patients [55]. Some of the associated features are corneal edema, band keratopathy, iridocorneal peripheral adhesions, iris atrophy, pupillary ectropion, and secondary glaucoma [56]. Jirsova et al. had reported several cytokeratins of which CK7 and CK19 have the most pronounced effect for the abnormal PPCD endothelium. Their study concluded that the pattern of cytokeratin expression found in the abnormal endothelium cells can be attributed to a metaplastic process during which endothelial cells are transformed into epithelial-like cells [57]. Merjava et al. reported changes not only in the endothelium and Descemet's membrane but also in the composition of the basal membrane epithelium and

the anterior stroma of PPCD corneas [58]. They found changes in collagen 4 and collagen 8 chains in PPCD corneas, which lead to increased proliferation of abnormal endothelium. Heon et al. had identified mutations in *VSX1* homeobox gene for both keratoconus and PPCD. Mutations in G160D and P247R led to detection of abnormal function of inner retina, the site for VSX1 expression [59]. But the study by Aldave et al. showed that the missense mutations Gly160Asp and Asp144Glu within the *VSX1* gene, were rare polymorphism and not disease causing mutations [60]. Another study determined that majority of the PPCD cases were caused due to mutations in gene encoding the two handed zinc finger homeodomain transcription factor TCF8 [61]. Although there are histologic similarities between PPCD and CHED, there are no reports on the evidence of oxidative stress in PPCD yet and detailed study needs to be done to have a clear understanding about the pathogenesis of the disease.

4. OXIDATIVE STRESS IN OTHER CORNEAL DISEASES

4.1. Keratoconus

Keratoconus is a progressive, non-inflammatory eye disease associated with protrusion and thinning of the cornea. The cornea weakens and assumes a conical shape with scarring and decreased vision [62]. It leads to blurry and double vision, near-sightedness, astigmatism, light sensitivity, and is usually bilateral. Keratoconus presents a complex etiology, as apart from environmental factors, genetics also plays an important role in manifestation of the disorder. Numerous studies have linked the role of oxidative stress in the pathogenesis of keratoconus [63, 64]. In healthy corneas aldehyde dehydrogenase (ALDH3) is found which absorbs UV rays and removes cytotoxic aldehydes produced by UV-induced lipid peroxidation [65]. Mice expressing enzymatically defective ALDH3 are more susceptible to the UV-induced pathology demonstrating the significance of ALDH3 [66, 67] which is found to be abnormal in keratoconus corneas [68]. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are reactive aldehydes which are produced during cell membrane destruction due to peroxidation of lipids as a result of increased ROS concentrations. It has been reported that cytotoxic byproducts such as superoxide, hydrogen peroxide, and hydroxyl radicals are extensively accumulated in keratoconic corneas due to abnormalities in their antioxidant signaling pathways [64]. Behndig et. al. found that the level of extracellular SOD is lower in keratoconus corneas when compared with normal healthy corneas [69].

Besides the antioxidant enzymes, the level of non-enzymatic antioxidants such as glutathione was also found to be lower in keratoconus corneas than in normal healthy corneas [70]. Corneal fibroblast cells taken from keratoconus patients show increased production of ROS and RNS. The presence of oxidative stress was indicated by nitrotyrosine, a marker for the formation of peroxynitrite, which showed elevated levels of ROS in keratoconus corneas [36]. Peroxynitrite and NO are responsible for various cytotoxic effects like DNA damage and activation of apoptosis [71]. The increase in tissue proteinase activities and the low levels of proteinase inhibitors due to oxidant products have been implicated for the thinning of cornea in keratoconus [72]. Elevated levels of ROS and RNS along with decreased antioxidant defenses in keratoconus corneas lead to degradation of extracellular matrix of the stroma and therefore causes thinning of stroma in keratoconus patients [64]. Altered expressions of oxidative phosphorylation proteins can lead to improper ATP synthesis and increased ROS and RNS formation. Very recently, Shetty et al. reported impaired regulation of autophagy due to oxidative damage in the cornea to be involved in the pathogenesis of keratoconus [73].

4.2. Granular Corneal Dystrophy Type 2

Granular corneal dystrophy type 2 (GCD2) is an abnormal condition due to granular and lattice type corneal deposits that causes age-dependent progression of corneal opacity leading to loss of vision. It is an autosomal dominant disorder caused by point mutation in *TGFB1* gene on chromosome 5q31 [74]. Choi et al. reported evidence of oxidative damage in the pathogenesis of GCD2 with increased levels of malondialdehyde and protein carbonyl groups. They also found increased level of catalase mRNA in GCD2 corneal fibroblasts compared to wild-type fibroblasts. Electron microscopy showed the presence of enlarged keratocytes and degenerated mitochondria in GCD2 corneas compared to control corneas [75, 76]. Morphological abnormalities in the meibo-

mian glands of these patients have also been reported [77]. Increased levels of intracellular ROS including H_2O_2 were also detected in GCD2 corneal fibroblasts compared to control fibroblasts [76]. Impaired autophagy is also linked with the pathogenesis of GCD2 [75]. These show the evidence of oxidative stress in GCD2 pathogenesis. The same group has also shown antioxidant melatonin was able to reduce the ROS level and increase the expression of melatonin receptors in GCD2 corneal fibroblasts, and thus, melatonin might have potential therapeutic implications for GCD2 treatment.

4.3. Schnyder Corneal Dystrophy

Schnyder corneal dystrophy (SCD) is an autosomal dominant inherited disease that is characterised by deposition of cholesterol and phospholipids which leads to progressive corneal opacity resulting in loss of vision. It is caused from a mutation in the *UBIAD1* (Ub1A prenyltransferase domain containing 1) gene located in chromosome 1. An almost ten-fold increase in cholesterol level has been reported in corneas with SCD [78]. Gatzioufas et al. reported the presence of lipid peroxidation and nitric oxide oxidation in SCD corneas compared to normal corneas that displayed minimal signal for nitrotyrosine [79]. They also reported increased level of MDA-thiobarbituric acid complex in aqueous humor of these patients. Involvement of mitochondrial UBIAD1 in cholesterol metabolism in SCD patients has also been reported. The interaction of UBIAD1 protein and apolipoprotein E outside mitochondria might also play an important role in the pathogenesis of SCD. Further studies are required to establish the mechanism of oxidative stress in the pathogenesis of SCD.

5. CONCLUSION

Recent ongoing studies have provided with many lines of evidence for oxidative stress playing an important role in the pathogenesis of corneal endothelial dystrophy. Smoking, automobile exhaust, industrial waste, blue light, and UV rays are all different factors which contribute significantly to the generation of oxidative stress in the eye. The increased generation of ROS and RNS tends to cause degeneration of corneal endothelial cells. Redox balance and activation of antioxidant signaling pathways in cornea help combat the oxidative stress. Antioxidant signaling pathways are not fully functional in corneas with different forms of endothelial dystrophies. Corneal transplantation is currently the only available mode of treatment, but surgical interventions are not always feasible, particularly in rural places due to non-availability of donor corneas. Developing new and better therapeutic approaches to curbing the damaging effect of oxidative stress is highly required. Activating the antioxidant signaling pathways mediated by Nrf2 can be an excellent pharmacological approach to treating corneal endothelial dystrophies.

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