

Oxidative stress in the trabecular meshwork (Review)

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Abstract. Glaucoma is the second leading cause of blindness worldwide and elevated intraocular pressure (IOP) is the most important risk factor. High IOP usually occurs as a result of an increase in aqueous humor outflow resistance at the trabecular meshwork (TM). An abnormal TM contributes to the development of glaucoma. Oxidative stress and vascular damage are considered two major cellular factors that lead to alterations in the TM. In this review, we discuss the findings related to oxidative damage to the TM, including the sources of oxidative stress in the TM such as the mitochondria, peroxisomes, endoplasmic reticulum, membrane, cytosol and exogenous factors. We also discuss antioxidants and clinical studies related to protection against oxidative stress in the TM. Although many questions remain unanswered, it is becoming increasingly clear that oxidative stress-induced damage to the TM is related to glaucoma. This may inspire new studies to find better and more stable antioxidants, and better models with which to elucidate the mechanisms involved, and to determine whether *in vitro* findings translate into *in vivo* observations. The regulation of the oxidative/redox balance may be the ultimate target for protecting the TM from oxidative stress and preventing glaucoma.

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1. Introduction

Glaucoma is the second leading cause of blindness worldwide (1). It is characterized by optic disk changes and progressive visual field loss, and eventually leads to the apoptosis of retinal ganglion cells and axon loss (2). Elevated intraocular pressure (IOP) is the most important risk factor for glaucoma (3,4). High IOP usually occurs as a result of an increase in the aqueous humor outflow resistance of the trabecular meshwork (TM). The TM is composed of trabecular beams made of extracellular matrix (ECM) elements, including fibronectin, laminin and collagen (5). Cells that line the trabecular beams are believed to be essential for regulating the aqueous humor outflow that controls IOP. TM abnormalities are the most common pathogenesis of glaucoma (6-9). Still, the pathogenesis of glaucoma is unclear as is the reason why the TM fails to maintain normal levels of aqueous humor outflow resistance. Oxidative stress and vascular damage (6) are considered two major alterations in the TM related to glaucoma (10). In this review, we discuss findings related to oxidative damage to the TM.

2. Oxidative stress

Free radicals are moieties with an unpaired electron and occur as normal metabolites. Under physiological conditions, cells produce up to 10^{11} free radicals per day. Free radicals may be classified as oxygen and non-oxygen moieties. Among these, oxygen radicals account for 95%. Oxygen radicals include oxygen and highly reactive oxygenated molecules, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\bullet), peroxide hydroxyl radicals, alkoxy radicals, superoxide and the anion radical (O_2^-), which are collectively known as reactive oxygen species (ROS) (11). Reactive nitrogen species also play an important role in oxidative stress (12); however, they will not be discussed in this review.

Under physiological conditions, the production and elimination of ROS are in equilibrium; however, some xenobiotics, ionizing radiation, illnesses, or aging may cause the production of ROS to levels that exceed the neutralizing capacity of an organism, thus leading to a series of pathological changes, which in turn leads to oxidative stress. This process is related to defense mechanisms, such as neutrophil inflammatory infiltrates, an increase in protease secretion and the generation of oxide intermediates. This process is similar to the normal aging process, although more severe (11). It is a prominent feature of many acute and chronic diseases (13).

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Oxidative stress plays an important role in pulmonary fibrosis, epilepsy, hypertension, atherosclerosis, Parkinson's disease and sudden death, and it is also known to be associated with a number of ophthalmic diseases, such as age-related macular degeneration, cataract and glaucoma (14,15).

3. Sources of ROS in the TM

ROS in cells are derived from both endogenous and exogenous sources. In the endogenous process, the majority of ROS are generated as a by product of normal metabolism (16). The sources of ROS are summarized in Fig. 1.

Mitochondrial ROS production. The mitochondria consume >90% of cellular oxygen in aerobic organisms under physiological conditions. Of this, approximately 1-5% of the oxygen is converted to ROS (17,18). In the mitochondria, the electron transport chain resides in the inner membrane where electrons are transmitted from NADH/FADH₂ to oxygen to produce H₂O. However, some electrons leak before they reach the final step, prematurely reacting with O₂ to form superoxide instead of H₂O (19-21).

Peroxisomal ROS production. Peroxisomes are monolayer vesicles, 0.5-1.0 μ m in diameter, that generally exist in eukaryotic cells. Peroxisomes contain a variety of enzymes, such as flavoenzymes and oxidoreductases. All these enzymes are either involved in the oxidation of fatty acids, D-amino acid catabolism and anabolism, glyoxylate/dicarboxylate metabolism, or in the production of spermidine, an autophagy-stimulating, life-prolonging substance. Peroxisomes produce H₂O₂ as a byproduct (18,22) and also produce O₂⁻, which mainly originates from the enzyme, xanthine oxidase, that is also found in the cytosol and is essential for purine degradation (23).

Endoplasmic reticulum ROS production. The mitochondria were believed to be the main producer of ROS in the cell; however, an increasing number of studies over the past decade have indicated that the endoplasmic reticulum, as well as peroxisomes produce as much or even more ROS than the mitochondria (18,22). In the endoplasmic reticulum, ROS are mainly produced by cytochrome P450 mono-oxygenases (P450), a superfamily of heme thiol proteins that are also distributed in the mitochondrial inner membrane. P450 is responsible for the synthesis and degradation of endogenous substances (i.e., fatty acids and hormones) and the detoxification of xenobiotics and lipophilic compounds. In this process, electrons are transferred from NADPH to cytochrome P450 via cytochrome P450 reductase, leading to the hydroxylation of xenobiotics. The leakage of electrons from this system can result in the formation of oxygen radicals, particularly O₂⁻ (24,25).

The endoplasmic reticulum is the main organelle responsible for protein processing. At the early stage of the protein unfolding process, the level of protein disulfide isomerase increases to correct misfolded proteins by forming correct disulfide bonds. Via the folding protein process, protein disulfide isomerase is reduced and an electron is transferred to molecular oxygen and glutathione (GSH). Incomplete transfer leads to the production of superoxide (26,27).

ROS produced in membranes and in the cytosol. Superoxide produced in mitochondrial and plasma membranes (29-31) is due to the activity of NADPH oxidases which is different from all other byproduct process. The superoxide produced in these membranes acts as a signaling molecule to protect against invading microorganisms (28). Electrons are passed on from NADPH to FAD to two b-type hemes and finally to O₂, resulting in the formation of superoxide.

In the cytosol, ROS are produced as a byproduct of arachidonic acid metabolism. With NADH or NADPH, superoxide is generated by cyclooxygenase and lipoxygenase enzymes that use arachidonic acid to synthesize prostaglandin H₂ or leukotrienes (32). Additionally, in the cytosol, ferrous iron reacts with H₂O₂ and, via the Fenton reaction, generates ferric iron, the very reactive hydroxyl radical (OH[•]), and hydroxide (OH⁻).

Exogenous factors. Infrared, ultraviolet (UV) and visible light can cause oxidative damage to the eye. UV light induces mutations that have been linked to a variety of ophthalmic pathological changes. UV light can be categorized by its wavelength as: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). All UVC and the majority of UVB light are absorbed by the cornea. Only UV wavelengths longer than 295 nm can be transmitted through the cornea to the anterior chamber; thus the aqueous humor and TM are only exposed to a small amount of UVA. However, even this small amount of UVA leads to the generation of ROS that are one of the main causes of oxidative stress in the TM (33,34). The UV-irradiation process can affect DNA, particularly mitochondrial DNA (mtDNA). It often leads to a 'common' 4977-bp long deletion of mtDNA that can increase mitochondrial ROS production. Increased levels of ROS can also lead to increased levels of mtDNA damage. Infrared light is absorbed by the mitochondrial electron transport chain, particularly at complex IV, leading to an increased leakage of ROS into the mitochondrial matrix. Besides, X-rays, some environmentally toxic moieties and some chemotherapies, as well as inflammation can cause oxidative damage (18,35,36).

Endogenous and exogenous factors together contribute to pathogenic events that set forth the process of oxidative stress-induced damage. Intense exposure to light, robust metabolic activity, and high oxygen tension are considered the major causes of pathological ROS (37).

The TM is surrounded by aqueous humour and thus the maintenance of the redox state of the aqueous humor is of vital importance to the TM. Inner TM cells resident near the anterior chamber are more severely exposed to oxidative damage (38,39). In the anterior chamber, H₂O₂ and other ROS are mainly generated by a light-dependent reaction with iris melanin (40). Metabolic pathways, inflammatory processes and phagocytosis are also important generating pathways. The concentration of H₂O₂ in the aqueous humor is believed to be 25 μ M (41).

4. Oxidative damage in the TM

Free radicals take part in many important life processes. They are closely related to cell proliferation, differentiation, apoptosis, muscle contraction, nerve conduction and gene expression, and act as second messengers in cell signal trans-

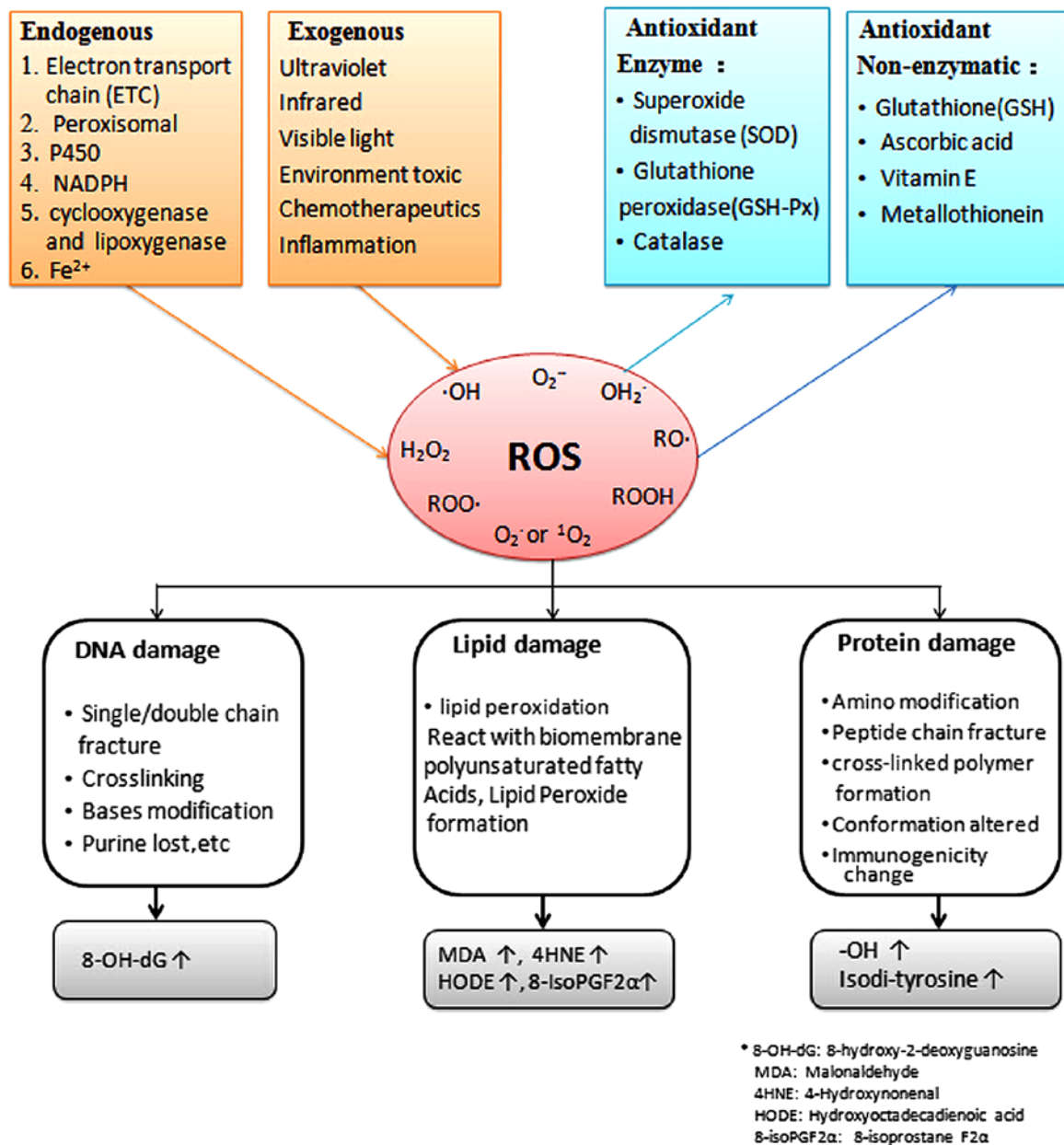


Figure 1. Factors associated with ROS production and cellular damage. ROS, reactive oxygen species.

duction (42,43). Due to or under some external causes, such as illness or aging, the oxidative balance of the body is compromised, leading to pathological changes. ROS damage proteins, lipids, and in particular, DNA molecules (44); these process are associated with the development of cell aging, chronic inflammation, cancer and apoptosis.

$\text{OH}\cdot$ is the most reactive ROS. It can react with different DNA moieties, including purine, pyrimidine and the deoxy-ribose backbone, resulting in irreversible mutations, such as single and double chain fracture, crosslinking between or within the chain, base modification and purine loss (16). mtDNA is less protected than nuclear DNA (45) and is more sensitive to oxidative stress (46). Superoxide anions mainly damage biological membranes, causing lipid peroxidation that generates cytotoxic secondary products of lipid oxidation. ROS also damage amino acid residues, particularly cysteine and

methionine residues, and damage the structure of critical areas, which leads to misfolding or dysfunction (47).

The TM is the most sensitive tissue to oxidative damage in the anterior chamber (48). Oxidative stress to the TM can cause much damage, such as reduce TM mitochondrial respiratory activity, leading to growth arrest (49), affect ECM structure (50) and lead to ECM accumulation (51), damage TM cellular DNA (52), alter membrane permeability (53), cause the rearrangement of TM cell cytoskeletal structures, cause the loss of cell-matrix adhesion (54), affect cell cycle progression (55), cause inflammatory cytokine release (56,57), and trigger apoptosis (58,59), as well as many forms of cell death (60). Cell death may cause a free radical attack (61,62) and the loss or altered functionality of TM cells, leading to even more oxidative stress, thus beginning a vicious cycle (63). At least, ROS alter the morphology, function and drainage of the anterior

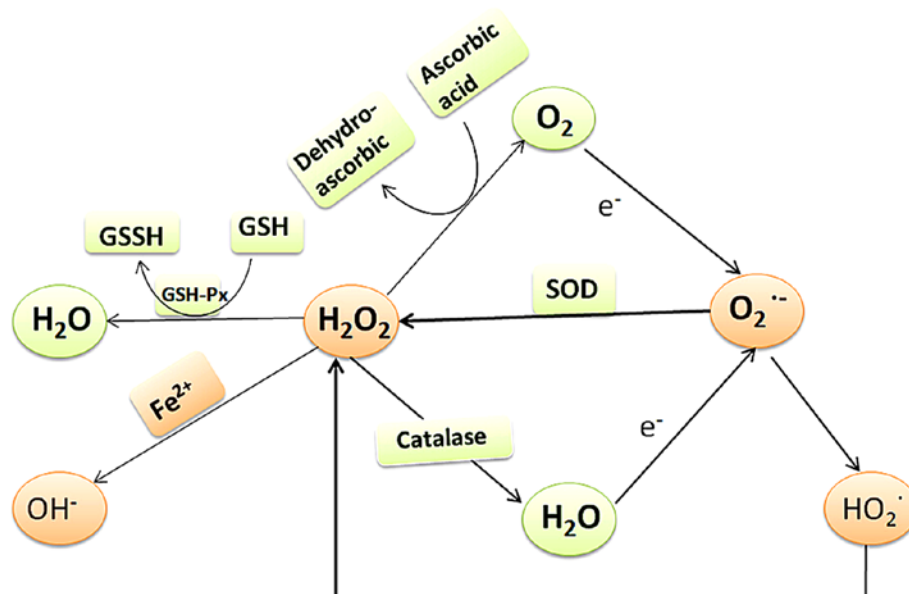


Figure 2. Mechanisms related to antioxidant activity. SOD, superoxide dismutase; GSH-px, glutathione peroxidase.

chamber filter channel that eventually leads to an increase in IOP (40,39,54,63-70). In patients with glaucoma, the levels of mtDNA damage and lipid peroxidation products in the TM are significantly higher compared with the controls (14,69,71) and their visual field defects, due to retinal ganglion cell degeneration, are directly proportional to oxidative damage to the TM (69,70).

5. Antioxidants in the TM

The ability of antioxidants in TM cells to counter oxidative damage is critical to their survival.

In a biological system, antioxidants can be categorized as enzymatic or non-enzymatic (Figs. 1 and 2). Antioxidant enzymes in the TM include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase and glutathione reductase (GSH-Re) (63,72). Non-enzymatic antioxidants include endogenously produced GSH or dietary compounds, such as vitamins C and E, and certain metal reduction proteins. The function of these molecules is to capture free radicals by accepting the unpaired electron and passing it on. In nocturnal animals, the levels of antioxidants in aqueous humor are much lower than in diurnal animals, suggesting that non-enzymatic antioxidants are consumed to protect the eyes from exogenous light damage (73-75).

In addition to the antioxidants mentioned above, TM cells have been shown to be able to synthesize a specific set of proteins, such as β -crystalline, that may act as molecular chaperones to prevent oxidative damage (76). Compared with plasma, the concentrations of ascorbic acid (530 μ M) and GSH (5.5 μ M) in aqueous humor are higher, which is important for maintaining the anterior chamber and TM oxidation balance (64). Ascorbic acid is considered to be the main antioxidant in the eye due to its high concentration in many ocular tissues (77-79). In the aqueous humor, the concentration of ascorbic acid is 15-fold higher than that in plasma (80). The mechanisms responsible for the antioxidant activity of ascorbic acid include the direct absorption of

UV light (81), quenching the fluorescence of biomolecules, and controlling fluorescence-mediated bio-transformations (82). A number of studies have demonstrated that the antioxidant activity of ascorbic acid depends on its concentration; ascorbic acid can also promote oxidation (83-85). Ascorbic acid can cause the decomposition of lipid peroxide and the generation of endogenous genotoxic substances; these substances can damage DNA and the level of these substance increases with the ascorbic acid concentration (86).

Oxidation and antioxidant systems in the eye cross-over to maintain balance. Classical examples include the GPX-GSH-GR-NADPH and GSH-vitamin C and E systems. These systems work together so a deficiency in one antioxidant is not always associated with eye pathologies (73). ROS production essentially depends on mitochondrial function and on the levels of antioxidant defenses (87). Age (88-90), diet and gene polymorphisms (91) also affect the ability of the body to resist and protect itself against oxidative damage.

In patients with primary open-angle glaucoma, the levels of circulating GSH are decreased, which indicates that the antioxidant defense system has been compromised (92). The levels of total reactive antioxidant potential and water soluble antioxidants, such as ascorbate and tyrosine in aqueous humor also decrease (93). The level of antioxidant enzymes in the aqueous humor of patients with primary open-angle glaucoma is controversial. Some articles have reported an increase in antioxidant enzymes (94,95), whereas others have reported a decrease (96,97). Whether the content of antioxidant enzymes correlates with the clinical course of primary open-angle glaucoma remains to be elucidated.

6. TM and oxidative stress *in vitro/in vivo*

Establishing a reliable oxidative stress model is essential to elucidating the mechanisms of oxidative stress and the efficacy of antioxidant drugs. In *in vitro* experiments, H_2O_2 is the most widely used agent in oxidative stress models. H_2O_2 can easily

pass through cell membranes and into cells, where it may react with iron ions to produce very active free radicals. In TM cells, the concentration of H_2O_2 usually ranges from 100 μM to 1 mM (54,57,98-100). Treatment concentrations and times vary significantly among different studies. In some studies, TM cells were exposed to 200 μM H_2O_2 for 30 min (57) or 300 μM for 1 h (103) which caused a 60% reduction in mitochondrial activity. In other studies, TM cells were exposed to 1 mM H_2O_2 for 24 h, resulting in a rate of cell death of approximately 50% (98,99). The difference here may be due to the instability of H_2O_2 . H_2O_2 is usually stable in solutions with a pH between 3.5-4.5; however, it easily decomposes in alkaline solutions or when exposed to bright light, particularly shortwave radiation. Tert-butyl hydroperoxide (tBHP) is a common lipid hydroperoxide. Unlike H_2O_2 , tBHP is not degraded by catalase, which allows it to cause oxidative stress for a longer period of time compared with H_2O_2 (101).

In the past, the degree of oxidative stress was usually measured by quantifying the activity of SOD, catalase and GSH-Px (93); however, currently the levels of products of oxidation such as oxidized lipids, proteins, amino acids and DNA are measured as they are more stable. Measured products include the lipid peroxidation products, hydroxyoctadecadienoic acid and malondialdehyde, and the DNA oxidative modification marker, 8-OH deoxyguanosine (Fig. 1) (11).

In TM cells, after exogenous oxidative treatment, the following damage has been observed: a decrease in cellular activity (102), a change in cell cycle progression, the inhibition of cell proliferation (103), and the promotion of cellular senescence (100). Oxidation treatment can rearrange the cell cytoskeleton structure (actin and vimentin) (54,102), increase the synthesis of ECM (fibronectin, plasminogen activator inhibitor 1, connective tissue growth factor) (103), decrease the adhesion to ECM (fibronectin, laminin, collagen types I and IV) (54), and increase the expression of some inflammatory mediators [interleukin (IL)-1 α , IL-6, IL-8 and endothelial-leukocyte adhesion molecule 1 (ELAM-1)] (57,103), leading to cell apoptosis and death (100). Nuclear factor (NF)- κB is the most relevant pathway associated with H_2O_2 -induced changes (54,103). NF- κB expression increases, and activate downstream target genes, including mitogen-activated protein kinase (MAPK) signaling pathways; phosphoinositide 3-kinase (PI3K)-Akt, extracellular signal-regulated kinase (ERK) and p38 have all been reported to contribute to cellular damage (101,102).

Oxidative *in vivo* models are diverse; however, few studies have examined oxidative stress in the TM. Non-specific methods include the use of irradiation, inhaled ozone and hypoxia-reperfusion, which may hardly reach effective concentrations in the TM during a short period of time. For the research of oxidative stress in the TM, methods involving the injection of drugs near targeted tissues, which have been widely reported in many other ocular tissues, such as the retina and lens (104,105) may be considered for future studies (106).

7. Clinical studies on protection against oxidative stress for the treatment of glaucoma

A series of substances have been reported to have potential antioxidant effects, such as creatine, α -lipoic acid, nicotinamide and catechins. These substances mainly include some antioxidant enzymes, oxidase inhibitors, vitamins C and E, some metal

ions such as Se and Zn, and some hormones. Some foods which have ingredients such as polyphenolic flavonoids (107) such as tea, coffee, dark chocolate (108), red wine (109), anthocyanosides (110) found in blueberries and *Ginkgo biloba* (111) also have antioxidant effects. However, all of these antioxidant substances lack targeting and specificity. There are some compounds that have been shown to protect TM cells from oxidative stress *in vitro* (103,112); however, their effects are limited *in vitro*. *In vivo*, dorzolamide, a carbonic anhydrase inhibitor, has been reported to reduce oxidative products and increase antioxidant enzyme activity in the aqueous humor of patients with primary open-angle glaucoma (113).

It is worth emphasizing that although oxidative stress has been confirmed to play a role in many diseases, antioxidant supplements are not always good for the health (114) and sometimes may even cause harm (115-117). In a 2-year randomized controlled trial, oral antioxidant supplementation in 117 patients with mild or moderate glaucoma had no effect (118), and some researchers have indicated that antioxidants promote cancer cell metastasis (119). As in studies of other systems, the research of antioxidant treatments for TM protection or glaucoma needs to be designed to elucidate how to use antioxidant compounds, determine when is the best intervention time (to prevent or to treat), and who (healthy or unhealthy individuals) can benefit from these compounds.

8. Conclusion and future perspectives

Although many questions remain unanswered, it is becoming increasingly clear that oxidative stress-induced damage to the TM is related to glaucoma, which may inspire further studies to find better and more stable antioxidants and better models with which to elucidate the mechanisms involved, and to determine whether *in vitro* findings can translate into *in vivo* observations. The regulation of the oxidative/redox balance may be the ultimate target for protecting the TM from oxidative stress and preventing glaucoma.

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