Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

Review

Oxidative stress, mitochondrial damage and diabetic retinopathy

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ARTICLE INFO

ABSTRACT

Diabetes has emerged as an epidemic of the 21st century, and retinopathy remains the leading cause of blindness in young adults and the mechanism of this blinding disease remains evasive. Diabetes-induced metabolic abnormalities have been identified, but a causal relationship between any specific abnormality and the development of this multi-factorial disease is unclear. Reactive oxygen species (ROS) are increased and the antioxidant defense system is compromised. Increased ROS result in retinal metabolic abnormalities, and these metabolic abnormalities can also produce ROS. Sustained exposure to ROS damages the mitochondria and compromises the electron transport system (ETC), and, ultimately, the mitochondrial DNA (mtDNA) is damaged. Damaged mtDNA impairs its transcription, and the vicious cycle of ROS continues to propagate. Many genes important in generation and neutralization of ROS are also epigenetically modified further increasing ROS, and the futile cycle continues to fuel in. Antioxidants have generated beneficial effects in ameliorating retinopathy in diabetic rodents, but limited clinical studies have not been encouraging. With the ongoing use of antioxidants for other chronic diseases, there is a need for a controlled trial to recognize their potential in ameliorating the development of this devastating disease.

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1. Introduction

Diabetes, a metabolic disease in which the ability of the body to properly utilize the glucose is impaired, is now emerging as an epidemic of the 21st century. Glucose utilization could be impaired either due to lack/reduction in insulin production (type I diabetes) or due to body's ability to utilize insulin (type II diabetes). Although hyperglycemia in these two forms of diabetes is the outcome of two different reasons, over time, it causes a number of health problems in both type I and type II diabetic patients. According to International Diabetes Federation, every 7 s a person dies from diabetes. With the number of patients afflicted with this chronic disease is rising at an alarming rate, diabetes has become a major public health issue. According to International diabetes federation, 382 million people worldwide have diabetes, and this number is projected to rise to 592 million in less than 25 years [1]. In this life-long disease, sustained high circulating glucose damages the vasculature resulting in chronic micro- and macro-vascular complications throughout the body. Damage to the macrovasculature increases the prevalence of heart disease, stroke and peripheral arterial disease, and to the microvasculature, in retinopathy, neuropathy and nephropathy in diabetic patients.

2. Diabetic retinopathy

Retinopathy, one of the major microvascular complications of diabetes, is considered as the most feared complications among the young adults, and it is the leading cause of acquired blindness in working age adults. Sustained hyperglycemia damages the microvascular bed of the retina, the innermost layer of the eye, ultimately impairing its ability to convert light signals. This is a duration-dependent disease, which is rarely detected within the first few years of diabetes, but its incidence dramatically increases with time, and after 20–25 years of diabetes almost 90% of patients present some signs of retinopathy [2]. With the recent advancement in blood glucose management and increase in awareness of the disease, the incidence and the risk of progression of diabetic retinopathy is on a descending end; the population-based Wisconsin Epidemiologic Study of Diabetic Retinopathy has documented 77% decrease in the estimated annual incidence of proliferative diabetic retinopathy between 1998–2007 [3]. However, with the increase in the incidence of diabetes at an alarming rate, the number of people with diabetic retinopathy is also projected to grow from 126.6 million in 2010 to 191.0 million by 2030. Based on current estimates, increase in the number with vision-threatening diabetic retinopathy is projected to increase from 37.3 million to 56.3 million [4], and it is critical to understand the mechanism of its development.

Microvasculature of the retina is the major site of pathology associated with diabetic retinopathy [2]. The retina, a light-sensitive layer of tissue which lines the inner surface of the eye, is the most metabolically active tissue in the body. Through a cascade of chemical and electrical reactions, it converts light into nerve impulses, and optic nerve

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transmits these signals to the brain. The retina has a complex structure with a number of layers and cells types. The major cell types in the retina are the vascular cells (pericytes and endothelial cells), macroglial cells (Muller cells and astrocytes), neurons (photoreceptors, bipolar cells, amacrine and ganglion cells), and microglia (which act as phagocytes). The vasculature of the retina occupies the inner half of the neural retina, and is a highly organized structure. The innermost portion of the retina contains the larger vessels and the space between the nerve fiber and inner nuclear layer is occupied by the microvasculature. The retinal capillaries are surrounded by a connective tissue sheath, the basement membrane, and inside the capillaries is a lining of endothelial cells. Endothelial cells form connections between each other, and these tight junctional complexes act as barrier between the retina and the blood circulation. Tight junctions impede the outward flow of macromolecules, and maintain the microenvironment [5]. However, in diabetes, disruption of blood-retinal barrier allows albumin and other macromolecules to leak out into the vitreous. The capillaries are covered by a layer of smooth muscle cells, pericytes; these cells help in contraction to regulate retinal vascular tone and blood flow. Diabetic environment thickens the basement membrane and accelerates the loss of pericytes and endothelial cells and damages the vasculature [2,6].

Diabetic retinopathy is a slow progressing disease, in the initial stages, it is asymptomatic and the patients may have 20/20 vision. In this non proliferative stage, microaneurysms (very small blood-filled bulges) are visible by fundus photography, and fluorescein angiography could show some leakage. The patients might experience thickening of the macula, and due to macular edema, vision becomes blurred. This nonproliferative stage of diabetic retinopathy, if not controlled, could progress to proliferative diabetic retinopathy, where neovascularization appears at the back of the eye and extend into the vitreous of the eye, and these new blood vessels begin to bleed scaring the vitreous body leading to retinal detachment, and ultimately to blindness [2].

Animal studies have shown that in the early stages of the disease, the basement membrane of the capillaries is thickened; pericytes are lost before any histopathological signs begin to appear. Loss of pericytes makes capillaries collapse, and endothelial cells begin to die [6]. Although impaired retinal function is reported in diabetic patients and in animal models of diabetic retinopathy before appearance of any histopathology characteristic of diabetic retinopathy, the disease has largely been related with the microvasculature of the retina. Recent work has suggested that the neuronal unit of the retina is also intimately associated with diabetic retinopathy as retinal neurons and glial cells also show biochemical defects and functional abnormalities, including accelerated apoptosis of neurons, activation of microglial cells and increased production of oxidative stress by photoreceptors [7]. Furthermore, with the worsening of diabetic retinopathy, these neuronal lesions also show progressive impairments. But, how these neuronal abnormalities contribute to the histopathology characteristic of diabetic retinopathy remains unclear.

3. Molecular mechanisms of diabetic retinopathy

Diabetic retinopathy is a multi-factorial disease with a complex etiology. Diabetes Control and Complications Trial (DCCT), and follow up Epidemiology of Diabetes Interventions and Complications (EDIC) studies, have clearly documented the importance of good glycemic control in inhibiting/preventing the development of diabetic retinopathy, and early intervention remains crucial to prevent its further progression [8]. Similar results are also demonstrated in *in vitro* and *in vivo* models of diabetic retinopathy [9–13]. However, maintenance of good glycemic control for this life-long disease is, at times, could be challenging, and unachievable, and to identify a therapeutic target, understanding the mechanisms becomes very important. Although hyperglycemia remains the main instigator of the development of diabetic retinopathy, other systemic factors, including dyslipidemia and hypertension are also associated with its development [14]. Despite extensive ongoing

research in the field to understand the etiology of this complex disease, the exact mechanism responsible for its development remains elusive.

In vivo and *in vitro* models of diabetic retinopathy have identified many metabolic abnormalities, and some of the major pathways include activation of advanced glycation end products (AGEs) formation, polyol and hexosamine pathways and protein kinase C (PKC), and increase in oxidative stress [15–19].

Diabetic environment facilitates the interactions of glucose with amino acids in proteins, lipids and nucleic acids, and *via* non-enzymatic reactions, form Schiff's base and Amadori products [19]. A complex cascade of reactions, ultimately results in the conversion of Amadori products into the formation of AGEs [20]. Increased AGEs and their receptors (RAGEs) are observed within retinal capillaries, and are associated with increased inflammation and capillary cell loss in diabetic retinopathy [21]. Furthermore, the gene encoding RAGEs is considered to serve as a surrogate marker for patients vulnerable to diabetic complications [22].

Excess glucose is converted to sorbitol by aldose reductase, an enzyme which uses nicotinamide adenine dinucleotide phosphate (NADPH) as a hydrogen donor and has a high 'Km' for glucose. Because of consumption of NADPH, its availability for the biosynthesis of intracellular antioxidant glutathione (GSH) is decreased. Sorbitol dehydrogenase further converts sorbitol to fructose by utilizing NAD^+ as a hydrogen donor [15]. Increased polyol pathway activity is seen in the retina and its capillary cells in diabetes, and genetic association between the C allele of the polymorphism at position − 106 in the promoter of aldose reductase gene *AKR1B1* and diabetic retinopathy [23]. However, the exact mechanism by which polyol pathway plays a role in the development of diabetic retinopathy remains elusive, and clinical trials using aldose reductase inhibitors have been inconclusive [24].

Increased levels of circulating glucose are shown to activate diacyl glycerol-PKC cascade, and PKC activation in retinal vasculature is implicated in increased vascular permeability, alterations in blood flow and stimulation of neovascularization seen in diabetic retinopathy [25]. PKC is also implicated in the acceleration of apoptosis of capillary cells, resulting in degenerative capillaries and pericyte ghosts, the early histopathological signs seen in animal models of diabetic retinopathy [25]. Thus, the importance of understanding how hyperglycemia alters multiple pathways through changes to only a few essential cellular elements is very critical. PKC inhibitor, ruboxistaurin, has shown encouraging initial results in attenuating retinal hemodynamic abnormalities in diabetic patients, clinical trials have not been very promising [26].

The flux of fructose 6-phosphate into the hexosamine pathway is also increased in diabetes, and activation of this pathway serves as an alternative to glycolysis to utilize overproduction of fructose 6-phosphate [15]. Activation of hexosamine pathway is implicated in apoptosis of retinal capillary cells in diabetes [27], and in limiting pericyte proliferation.

Circulating high glucose is closely related to increase in oxidative stress, and the retina being a tissue rich in polyunsaturated fatty acids with high glucose oxidation and oxygen uptake [28], is considered as a good target of increased oxidative stress in diabetes. Increased oxidative stress, the major focus of this review, is postulated in the development of major diabetic micro- and macro-vascular complications of diabetes. Increase in oxidative stress is associated with a wide range of microvascular abnormalities associated with the development of diabetic retinopathy including, cell loss, hemodynamic and structural changes and basement thickening [15–18,29,30]. Increased serum lipid hydroperoxides are also associated with increased prevalence of retinopathy in diabetic patients [31]. In addition to circulating high glucose itself, reactive oxygen species (ROS) are also produced by other diabetes-induced metabolic abnormalities including activation of PKC and AGEs [15,32].

Activation of these pathways is considered to stem from hyperglycemia and the accumulation of various glycolytic intermediates. A link

between any specific metabolic abnormality and the development of diabetic retinopathy, however, remains largely speculative, raising the possibility that no single metabolic abnormality could be responsible for development of this complex disease.

4. Oxidative stress

Normal cellular metabolism continuously produces free radical; the body utilizes ~95% of them for metabolism and 5% of the oxygen is converted into ROS. These ROS act as double edged sword; they can work as messengers in redox signaling, but can also damage normal cellular signaling. A very efficient antioxidant defense system with enzymatic and non-enzymatic antioxidants effectively detoxifies these dangerous ROS. However, under pathological conditions, production and detoxification of free radicals is impaired, due to either increased ROS production or decreased removal, or both, resulting in an imbalance between production and destruction of ROS create an excessive bioavailability of ROS. If these damaging free radicals are not readily neutralized, they damage macromolecules-proteins, lipids and DNA, and alter the expression of various stress-response genes further stimulating additional ROS generation from endogenous sources [33]. As mentioned above, enzymatic and nonenzymatic antioxidants are available to defend from the damaging effects of ROS. In diabetic retinopathy, the retina experiences both, increased production and decreased removal of free radicals [34].

5. Sources of oxidative stress

Both enzymatic and non-enzymatic mechanisms produce superoxide radicals, while cytosolic NADPH oxidase (Nox) is the primary enzyme responsible for their production [30], non-enzymatic production is mainly the consequence of mitochondrial respiration. Nox, a multiprotein enzyme, catalyzes one-electron reduction of oxygen to superoxide anion, and the process involves oxidization of cytosolic NADPH to NADP. In diabetes, increased activity of Nox is observed in many tissues, including pancreatic beta cells and retina, suggesting it to be one of the major sources of ROS generation [17,30].

Arginase is another important instigator of oxidative stress as increased arginase activity causes endothelial nitric oxide synthase (eNOS) uncoupling due to decreased levels of L-arginine, and uncoupled eNOS produces superoxide which reacts with available nitric oxide to form peroxynitrite. In diabetes, increased arginase in the retina is shown to be associated with endothelial cell dysfunction [35]. Furthermore, peroxynitrite, *via* protein tyrosine nitration, can also enhance the activity of NADPH oxidase, which can further fuel to increased ROS production.

6. Mitochondria and generation of superoxide

Mitochondria are the major source of free radicals; in normal physiological conditions, this account for approximately 2% of the total oxygen uptake. Electron transfer through complexes I, III and IV of the electron transport chain pump protons into the inter membrane space, generating a proton gradient that drives ATP synthase, but some electrons leak out from complex I or complex III, and interact with molecular oxygen to form superoxide anion [36]. Furthermore, acyl-CoA dehydrogenase and glycerol phosphate dehydrogenase also generate ROS [37], and increased glucose-derived pyruvate is oxidized in the tricarboxylic acid cycle, which increases the flux of electron donors into the electron transport chain. This overwhelms the electron transport chain and blocks the electron transfer inside complex III, causing the electrons to back up to coenzyme Q, and increasing free radical levels [38]. In the pathogenesis of diabetic retinopathy, inactivation of complex III activity is implicated in increased mitochondrial superoxide accumulation in the retina [39]. Furthermore, cytosolic ROS, *via* activation of matrix metalloproteinases [40], damage retinal mitochondrial membrane, and further acerbate mitochondrial dysfunction [41]. Thus,

ROS production by cytosolic and mitochondrial sources in a diabetic environment appears to be closely interrelated.

7. Oxidative stress and other metabolic abnormalities

Increase in oxidative stress in diabetes and its role in the development of diabetic complications is now well accepted. Many molecular mechanisms are implicated in the induction of increased oxidative stress, including auto-oxidation of glucose, impaired antioxidant defense enzymes, metabolic abnormalities initiated by hyperglycemia and damage to the mitochondria [2,15,17]. Furthermore, since diabetes is a life-long disease, continuing cycle of metabolic stress and tissue damage further increases free radical production. The retina and its cells experience increased oxidative stress in diabetes, and their mitochondria are damaged, and metabolic abnormalities, initiated in diabetic environment are also considered as potential contributor of increase oxidative stress. However, how these mechanisms lead to increased oxidative stress and to the development of diabetic retinopathy remains unclear.

In diabetes, activation of the polyol pathway has potential to increase oxidative stress *via* both, decreasing intracellular antioxidant and increasing production of ROS. Increased consumption of NADPH by aldose reductase, the rate limiting enzyme of polyol pathways, is associated with decreased GSH levels, and increased availability of NADPH for Nox, increases the generation of intracellular oxidant species [42].

Increased accumulation of AGEs is implicated in alterations in function, activity, and degradation of both intracellular and extracellular proteins *via* chemical rearrangement and cross-linking. AGEs accumulation within retinal capillary cells, induce permanent abnormalities in extracellular matrix component function and, *via* their recognition by receptors, causes oxidative stress by depleting GSH and reducing glyoxalase-1 recycling [43]. AGEs modify macromolecules and form intra-molecular and intermolecular cross-links, and increased interactions of AGEs with their receptors, RAGEs, can increase nitrative stress in the retinal vasculature [19]. In addition, AGEs, by a sequence of molecular and biochemical pathways, activate nuclear translocation factor-kB (NF-kB) and caspase-3, resulting in capillary cell apoptosis. Nitration of proteins may also disrupt protein assembly and functions, and can cause endothelial and neuronal cell apoptosis, ultimately leading to pathological consequences and damage of cellular constituents [44]. Thus, there appears to be a close loop between AGEs and oxidative stress.

While ROS can activate PKC, PKC activation can also result in the loss of thiols. Modification of PKC by increased oxidative stress can further contribute to redox-mediated signaling events, and prolonged oxidant exposure could result in sustained PKC activation [45]. In contrast, inhibition of PKC inhibits the hyperglycemia-induced increase in free radical production [32].

Activation of hexosamine pathway, as an alternative to glycolysis for the utilization of hyperglycemia-induced overproduction of fructose-6-phosphate, is also associated with endothelial cell and retinal neuron apoptosis [46].

Many inflammatory mediators and leukocyte adhesion are upregulated in diabetes, and diabetic retinopathy is now considered as a low grade chronic inflammatory diseases [47]. Increased ROS stimulate release of inflammatory cytokines *via* activation of NF-kB, and the levels of NF-kB are elevated in the retina and vitreous of patients with proliferative diabetic retinopathy and in animal models of diabetic retinopathy [48,49]. Cytokines themselves are also a good source of ROS production, and ROS are a strong stimulus for the release of the cytokines, thus suggesting a bidirectional mechanism [50,51]. In support, antioxidants administration to diabetic rats inhibits increase in retinal pro-inflammatory cytokines, and administration of cytokines in the vitreous of normal rats, increases oxidative damage [52].

From the literature cited above, diabetes-induced major metabolic abnormalities and oxidative stress appear to be inter-related where

increased oxidative stress fuels into the metabolic abnormalities, and metabolic abnormalities produce increased oxidative stress (Fig. 1).

## 8. Antioxidant defense system

To preserve the cellular redox homeostasis, and keep the free radicals in check, the cell has an efficient enzymatic and non-enzymatic antioxidant defense system. This antioxidant defense system is comprised of intracellular antioxidants and enzymes, including superoxide scavenging enzymes, glutathione peroxidase and reductase [34,53]. Intracellular antioxidants, such as glutathione and ascorbic acid neutralize the detrimental effect of free radicals, and enzymes scavenge free radicals, including superoxide radicals generated in the mitochondria, which do not readily cross the membranes. Manganese superoxide dismutase (MnSOD), a superoxide scavenging enzyme, converts intramitochondrial superoxide to hydrogen peroxide, which can diffuse out of mitochondria or further catalyzed into harmless byproducts and protect mitochondrial damage [54]. Regulation of mitochondrial function is controlled by nuclear-mitochondrial anterograde signaling, the signaling which regulates the expression of nuclear encoded stress responsive genes, forming cellular antioxidant defense system that help and protect mitochondrial function [55,56]. The cell is also equipped with a master transcription factor, NF-E2-related factor 2 (Nrf2). Nrf2 regulates the transcription of the enzyme critical in antioxidant defense, including glutamate cysteine ligase for GSH biosynthesis. In diabetes, the retina experiences compromised antioxidant defense system; the activities of antioxidant defense enzymes-MnSOD, glutathione peroxidase and catalase are decreased, the transcriptional activity of Nrf2 is also subnormal and mitochondrial function is compromised [57]. Furthermore, the levels of glutathione become subnormal [10]. The role of oxidative stress in the development of diabetic retinopathy is further strengthened by the studies showing decreased expression of thioredoxin-interacting protein, an endogenous inhibitor of thioredoxin [58], further adding an oxidative burden to the retina.

9. Mitochondrial damage

Mitochondria, the powerhouse of the cell, are the major endogenous source of free radicals, but are also the targets for its damaging effects. In the pathogenesis of diabetic retinopathy, mitochondria in retinal endothelium are swollen with partial crystolysis, and they are dysfunctional, with impaired respiration [39,41,59–61]. The role of mitochondria in the development of diabetic retinopathy is further supported by our studies showing the protection of the development of diabetic retinopathy in mice overexpressing the gene encoding mitochondrial superoxide dismutase, MnSOD [39]. Although it is clear that mitochondria play a role in the development of diabetic retinopathy, how diabetic environment damages them remains unclear, and below are some of the possible mechanism that could be function.

Experimental models (both in vitro and in vivo) have clearly shown that generation of ROS in hyperglycemic environment is an early event, but despite this increase, increase in ROS and damage to the
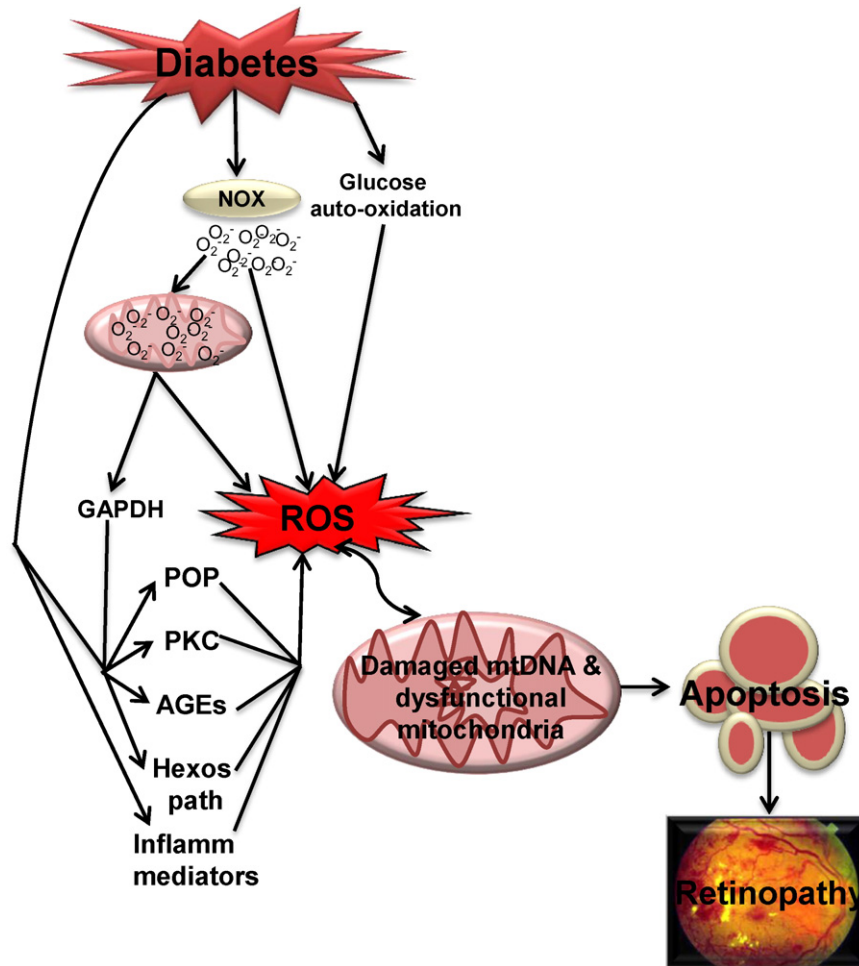
Fig. 1. Diabetic environment increases production of reactive oxygen species (ROS) and also induces many metabolic abnormalities, including activation of polyol pathway (POP), protein kinase C (PKC), advanced glycation end products (AGEs) and hexosamine (Hexos) pathway, and production of inflammatory mediators. Increased ROS damage mitochondria, and mitochondrial ROS, *via* inhibiting glyceraldehyde 3-phosphate dehydrogenase, feed into the metabolic abnormalities. Sustained accumulation of ROS damages mitochondrial DNA (mtDNA) and function, which subsequently accelerates cell apoptosis, and leads to the development of retinopathy.

mitochondria are not seen till the duration of hyperglycemic insult is extended [59,61]. The possibility that cytosolic ROS, *via* damaging the membrane structure of the mitochondria, decrease the mitochondrial membrane potential [62], however, cannot be ruled out. In support, we have shown that ROS produced in diabetic conditions progressively damage the mitochondria, and that could be contributing to the time lag between increase in cytosolic ROS and mitochondrial dysfunction seen in the retina and its capillary cells [17]. In diabetic retinopathy, activation of redox-sensitive gelatin matrix metalloproteinases (MMPs) is an early event, which is followed by their accumulation in the mitochondria, resulting in damage of endothelial mitochondria [41]. In addition, increased lipid peroxidation can also damage mitochondrial membrane, and retinal lipid peroxide levels are elevated in diabetes.

Increased oxidative stress can also activate redox-sensitive NF-*k*B, and its activation in diabetes in the retinal endothelial cells and pericytes is associated with their accelerated apoptosis [48]. Due to activation of NF-*k*B, the expression of the inducible form of nitric oxide synthase is increased resulting in increased nitric oxide production. Peroxynitrite, formed by reaction between nitric oxide and superoxide radicals interacts with mitochondria resulting in mitochondrial transition pore opening. This allows the release of cytochrome c, and activates the apoptotic machinery [59]. Peroxynitrite levels are elevated in retina early in diabetes and remain elevated at 14 months of diabetes in rats, and increased nitrotyrosine can be localized in the retinal vasculature of diabetic rats.

The coupling between the electron transport chain and ATP synthesis is mediated *via* the proton carriers, the uncoupling proteins (UCPs), and mild uncoupling of the inner mitochondrial membrane controls the membrane potential and regulates mitochondrial ROS [63]. Recent studies have shown an association between UCP1-3826A/G polymorphism and retinopathy in type I diabetic patients [64]. However, the exact role of UCPs in mitochondrial homeostasis in the development of diabetic retinopathy remains to be explored.

Mitochondria are also equipped with their own circular DNA, mtDNA, and this small DNA is in the close proximity to the electron transport machinery and lacks histones, making it prone to increased oxidative damage [65]. The activity of Complex III of the electron transport chain, the major source of superoxide production, is impaired in the retina and its capillary cells in diabetes [39]. Diabetes is shown to increase the levels of oxidatively modified guanine bases (8-OHdG) in the retinal mitochondria [66]. In addition, the number of sequence variants is increased [60]. Although mtDNA encodes only 13 proteins, these are essential for proper functioning of the electron transport chain and for the mitochondrial homeostasis [65]. Due to damage of mtDNA, its transcription is impaired, which further compromises the function of the electron transport, and the process results in increased ROS production [66]. Mitochondria are equipped with efficient DNA repair machinery, which consists of nuclear DNA-encoded base excision repair (BER) [67] and mismatch repair (MMR) enzymes. Although BER system recognizes, excises and replaces small base modifications in the DNA to repair the oxidative damage [67], the MMR system repairs uncomplimentary base pairs incorporated into the DNA. Diabetic environment attenuates the ability of this machinery to repair the damage, and one of the reasons appears to be the diabetes-induced poor ability of the mitochondrial transport system to transport these repair enzymes inside the mitochondria [68].

The essential transcription elements and the control sites for replication of mtDNA are localized on a non-coding region of the mtDNA, the *D-loop* region. We have shown that in diabetes, this unwound region is highly vulnerable, and is damaged more extensively than rest of the circular mtDNA [69]. Due to damage to the main transcription site of the mtDNA, transcription of the genes responsible for the electron transport is decreased, resulting in increased production of superoxide radicals. To make the bad situation worse, the binding of mtDNA replication enzyme polymerase gamma (POLG) at the *D-loop* is also attenuated, and mtDNA copy numbers are decreased [70]. Thus, suboptimal mtDNA transcription and biogenesis decrease the activity of the electron transport chain and increase superoxide radicals, and these superoxide radicals continue to fuel into the vicious cycle of free radicals. As diabetic retinopathy is a progressing disease, we have shown that during the initial stages of the disease, the transcription of the genes important in mtDNA biogenesis and repair is increased to overcome diabetes-induced increase in ROS, but as the disease progresses, the system gets overwhelmed, and mtDNA biogenesis and the repair mechanisms fail to keep up with the demand, and break apart [61]. However, regulation of increased mitochondrial superoxide accumulation by overexpression of the gene encoding mitochondrial superoxide dismutase, *Sod2*, in mice, which protects from the development of diabetic retinopathy, also prevents decreased mtDNA transcription and replication, suggesting a critical role of mitochondrial homeostasis in the development of diabetic retinopathy [39,69,71] (Fig. 2).

10. Epigenetic modifications, oxidative stress and diabetic retinopathy

In the pathogenesis of diabetic retinopathy, the expression of many genes associated with various metabolic abnormalities including inflammation and oxidative stress, are altered in the retina and its capillary cells [16,57]. Emerging studies have now documented that the gene expression is also regulated by the environmental factors and life style, and epigenetic modifications are now implicated in many chronic diseases. These epigenetic changes, without changing the DNA sequence, can control gene activity, and this dynamic process of transcriptional activation or repression depends on the recruitment of protein complexes that alter chromatin structure *via* enzymatic modifications of histone tails and nucleosome remodeling, altering the accessibility of transcription factors to the DNA [72]. Recent studies have suggested that these epigenetic changes could explain the chronic and persistent microvascular complications of diabetes, including retinopathy [11,12,18,58,70,73,74]. Recent studies have shown an association between the polymorphism in the gene that encodes histone methyltransferases, *SUV39H2*, and diabetic retinopathy [75]. Others have also shown significant increase in retinal histone acetylation in diabetes [76]. Thus, the data implicating the role of epigenetics in the development of diabetic retinopathy remains ambiguous.

Contrary to the clinical data, experimental models have clearly demonstrated the role of epigenetics in diabetic retinopathy, especially, in the regulation of oxidative stress. Our studies using *in vitro* and *in vivo* models of diabetic retinopathy have shown *Sod2*, is epigenetically modified with increased methylation of H4K20 and acetylation of H3K9, facilitating the binding of NF-*k*B at its promoter/enhancer [74]. In addition, the function of the master regulator, Nrf2, is also affected by the epigenetic modification, we have shown that the histones at the promoter of the intracellular inhibitor of Nrf2, Kelch-like ECH associated protein 1 (*Keap1*), are modified, and this alters the binding of transcriptional factor Sp1 [12]. Increased Keap1 compromises the transcriptional activity of Nrf2 by restraining it in the cytosol. Histones at the promoter of the enzyme critical in biosynthesis of the intracellular antioxidant GSH are also modified in diabetes, resulting in decreased binding of the transcriptional factor Nrf2, and decreasing its transcription [11,12,77]. In addition, in diabetes, thioredoxin-interacting protein is shown to promote epigenetic alterations at the promoter of retinal cyclooxygenase-2 [58]. Thus, histone modifications appear to play an important role in the maintenance of the antioxidant defense system of the retina in diabetes. Moreover, epigenetic modifications also facilitate the damage to the retinal mitochondria; hypo-methylation of H3K9, with concomitant hyper-acetylation of H3K9 at retinal *MMP-9* promoter is also implicated in its activation of in diabetes, and *MMP-9* is shown to initiate the apoptotic machinery by damaging the mitochondria [78].

In addition to histone modifications, methylation status of the cytosine in the DNA molecule also regulate gene transcription as DNA is not
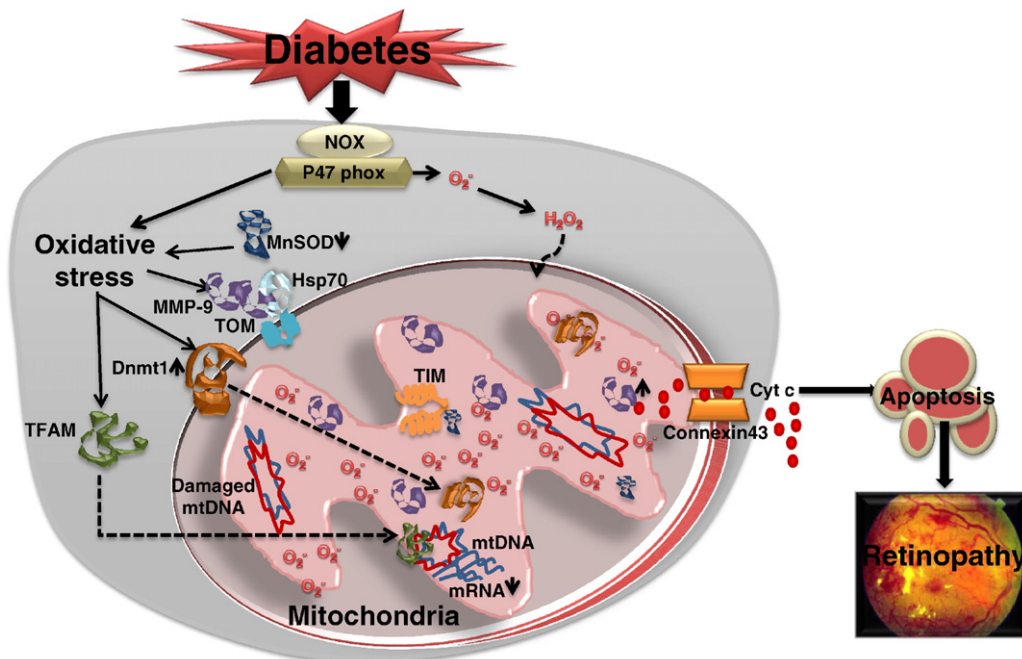
Fig. 2. Diabetes activates NADPH oxidase (NOX) complex, and increased oxidative stress alters the cellular gene expression, including that of matrix metalloproteinase 9 (*MMP-9*), mitochondrial superoxide dismutase (MnSOD), DNA methyltransferase 1 (Dnmt1) and mitochondrial transcription factor A (TFAM). Due to abnormalities in the chaperons (Heat shock protein, Hsp70) and mitochondrial membrane transport system (TOM and TIM), mitochondrial accumulation of Dnmt1 and *MMP-9* are increased and that of TFAM is decreased. Increased mitochondrial *MMP-9* damages mitochondrial connexin 43, and increases pore permeability, facilitating the leakage of cytochrome c (Cyt c) into the cytosol and activating the apoptotic machinery, and decreased accumulation of TFAM in the mitochondria impairs mtDNA biogenesis. Due to increased Dnmt1 in the mitochondria, mtDNA is hypermethylated and its transcription is impaired, and this compromises the electron transport system generating more free radicals. Due to cytoplasmic degradation of TFAM by oxidative stress, its mitochondrial levels are decreased and mitochondrial biogenesis is impaired, and this further fuels into the vicious cycle of ROS. To make the bad situation worse, the activity of mitochondrial scavenging enzyme is also decreased, and free radicals continue to feed into the vicious cycle.

a static, and this 'highly dynamic' entity responds to the environmental stimuli by modifying its properties in adapting to the changes. Methylation of the CpG changes protein-DNA interactions leading to alterations in chromatin structure, and this interferes with the binding of transcriptional machinery, resulting in gene suppression [79]. The methylation process is carried out by DNA methyl transferases (Dnmts), a family with 5 members, out of which only Dnmt1, Dnmt3a and Dnmt3b are catalytically active. Diabetes activates Dnmts in the retina, and CpG islands at the regulatory region of *POLG* are hypermethylated decreasing its binding with the *D-loop* region of mtDNA, interfering with mtDNA biogenesis [70]. In addition to the nuclear DNA, mtDNA is also susceptible to epigenetic modifications as this small DNA has ~450 CpG sites and ~4500 cytosine residues at non-CpG sites. Increased mtDNA methylation is observed in chronic diseases, including cancer [80], and our recent studies have shown that in diabetes, increased Dnmt1 expression in the retinal mitochondria hypermethylates its mtDNA [81]. Due to hypermethylation of the *D-loop* region, mtDNA transcription is impaired and electron transport chain is compromised, and mitochondrial homeostasis continues to disturbed. Thus, although mtDNA methylation field is still in its infancy, it holds a good promise for understanding the pathogenesis of diabetic retinopathy.

In addition to histone modifications and DNA methylation, small non-coding RNAs (miRNAs) are also altered in diabetes, and these miRNAs can regulate post-transcriptional gene expression by binding to their target messenger RNAs. Models of diabetic retinopathy have shown that upregulation of vascular endothelial growth factor (VEGF) is associated with the downregulation of *miR-126*, *miR-146a* and *miR-200b* and upregulation of *miR-195* downregulates Sirt1 [82,83]; as mentioned in the previous section, Sirt1 plays an important role in regulation of NF-*k*B, a transcriptional factor associated with the regulation of *MMP-9* and Sod2 [74,78,84].

Thus, the role of epigenetic modification is now becoming more evident than previously appreciated.

11. Therapeutic implications

Here we have presented overwhelming evidence that oxidative stress is increased in the retina in diabetes, and increased oxidative stress plays a major role in the development of diabetic retinopathy. As in diabetic environment multiple mechanisms can increase oxidative stress, and different antioxidants can either inhibit the production of ROS, or neutralize superoxide radicals, or augment antioxidants defense system, many antioxidants have been tried in animal studies.

Administration of green tea, which is rich in polyphenols, in addition to inhibiting increased retinal ROS levels, also provides neuroprotection, and ameliorates diabetes-induced abnormalities in blood-retinal breakdown and electroretinograms [85]. It is also shown to attenuate histopathology characteristic of diabetic retinopathy, including the formation of degenerative capillaries and formation of pericyte ghosts.

Lipoic acid, an organosulfur compound capable of thiol-disulfide exchange and scavenging ROS, decreases retinal levels of VEGF, 8-OHdG and nitrotyrosine and ameliorates NF-*k*B activation in diabetic rats [86]. Lipoic acid is also considered as a mitochondrial function and named them "mitochondrial nutrient", as it can directly or indirectly protect oxidative damage to the mitochondria and helps glutathione to maintain a healthy cellular redox state, and its administration prevents mitochondrial dysfunction and capillary cell apoptosis and the development of diabetic retinopathy in rats [86].

Benfotiamine, a lipid soluble thiamine (vitamin B1), prevents hyperglycemia-induced pericyte-endothelial cell interactions [87]. In diabetic rats, Benfotiamine has been shown to inhibit the development of histopathology characteristic of diabetic retinopathy *via* inhibiting the major biochemical pathways implicated in its pathogenesis [88].

Administration of nicanartine, an antioxidant which also has lipid-lowering properties, to diabetic rats is shown to provide partial benefit to the development of diabetic retinopathy; it inhibits the pericyte loss in the retinal vasculature, but does not reduce the formation of microaneurysms and acellular capillaries [89]. Trolox, a water soluble analog of vitamin E, reduces membrane lipid peroxidation and partially prevents the loss of pericytes in diabetic rats *via* reducing membrane lipid peroxidation [90]. However, no additional follow up studies have been reported for, both nicanartine and trolox.

Supplementation of rat diet with vitamins C and E prevents increase in oxidative stress in the retina by ameliorating decrease in the antioxidant defense machinery, and also decreasing the production of superoxide radicals. This amelioration in retinal oxidative stress is accompanied by decreased formation of both degenerative capillaries and pericyte ghosts in the retinal vasculature [34,53]. However, when vitamins C and E administration is supplemented by other antioxidants, including Trolox, N-acetyl cysteine, β-carotene and selenium, this multi-antioxidant mixture prevents activation of PKC, reduces oxidative stress and ameliorates the formation of microvascular lesions in the retina, the multi-antioxidants abrogate the diabetes-induced increases in retinal PKC and nitric oxide [53]. A carotenoid rich diet, which is in clinical trials for 'Diabetes Vision Function', prevents retinal capillary cell apoptosis and vascular and functional damage in diabetic rats [91]. Administration of the nutritional antioxidants that have been demonstrated to slow the progression of age-related macular degeneration in patients, protects the retina from diabetes-induced increased oxidative damage and prevents the development of retinal histopathology characteristic of diabetic retinopathy in rodents [92]. These results from experimental model have clearly demonstrated that this multi-factorial disease responds better to the diverse antioxidant mixture than just the vitamins C and E.

Literature cited above clearly suggest the importance of mitochondrial homeostasis in the development of diabetic retinopathy; and we have shown that overexpression of *Sod2* in mice prevents diabetes-induced mitochondrial damage in retinal capillary cells, including biogenesis and dysfunction, and also protects the retinal vasculature from the development of histopathology [39]. MnSOD mimics, Tempol and M40403 are shown to ameliorate endothelial dysfunction and abnormalities in endoneural blood flow [93]. We have shown that MnSOD mimic, MnTBAP, protects cells [59]. Suppression of *MMP-9* in mice protects diabetes-induced damage to the retinal mitochondrial structure and function, and ameliorates the formation of degenerative capillaries in the retinal vasculature [41]. In addition, inhibition of Nox2 activation by regulation of its upstream signaling axis, Tiam1-Rac1, ameliorate glucose-induced capillary cell apoptosis in retinal endothelial cells [17]. Thus, prevention of mitochondrial damage by suppression of the damaging *MMP-9*, or by preventing the accumulation of superoxide radicals by overexpressing *Sod2* protects the development of retinopathy in rodents.

Administration of curcumin, a polyphenol with potent antioxidant and anti-inflammatory effects, or Zeaxanthin, a dietary carotenoid, which is specifically concentrated in the retina, is shown to attenuate increase in retinal oxidative stress and proinflammatory markers in diabetic rats [94,95]. Peroxynitrite decomposition compound, FP15, ameliorates diabetes-induced increased leukocyte entrapment in the retinal microcirculation [96], and PARP inhibitor PJ-34, prevents the early lesions of diabetic retinopathy [97].

Although there is a wealth of data supporting the beneficial effects of antioxidants in the development of diabetic retinopathy, clinical studies have yet to demonstrate their benefits. Vitamin E is shown to ameliorate retinal hemodynamic abnormalities in diabetic patients [98], and oral administration of α-lipoic acid with genistein and vitamins for 30 days is shown to improve ERG oscillatory potential in patients with proliferative diabetic retinopathy [99]. Antioxidant properties containing calcium dobesilate and pycnogenol reduce the progression of diabetic retinopathy [100,101]. Our recent double masked study using a multi-component nutritional formula (vitamins C, D3, and E, zinc oxide, eicosapentaenoic acid, docosahexaenoic acid, alpha-lipoic acid, coenzyme Q10, zeaxanthin, lutein, benfotiamine, N-acetyl cysteine, resveratrol, turmeric root extract, green tea leaf) has shown encouraging results, as this multi-component nutritional formula can improve visual function and reduce serum inflammatory proteins in diabetic patient [102]. However, others have not observed any significant association between serum levels of major dietary antioxidants and retinopathy [103]. Also, a retrospective study in type II diabetic patients, based on a single 24-hour diet recall, fails to show any association between antioxidant supplementation (vitamins C and E, and β-carotene) and decrease in the severity of retinopathy [104].

Thus, despite encouraging results from the animal models, due to lack of well-designed longitudinal cohort studies, clinical studies have been inconclusive. This discrepancy could be the time of intervention for this slow progressing disease as DCCT/EDIC studies have shown the importance of the prior damage to the retina in the outcome of the tight glycemic control [8] Some of the other factors including the dose of the antioxidants and the duration of their administration in diabetic patients, could also influence the clinical outcome. In addition, although vitamin E is an antioxidant, it could also exhibit pro-oxidant effects and further acerbates oxidative stress [105]. Also the possibility that due to a well-defined blood-retinal barrier, transport of these antioxidants to the retina could be a limiting factor, cannot be ruled out. Despite these limitations, we believe that encouraging animal results and a very limited clinical data, strongly suggests for some longitudinal cohort studies and clinical trials.

Evidence provided above clearly shows the importance of epigenetic modifications in the development of diabetic retinopathy. Most of the inhibitors for histone acetylation have strong antioxidant properties. Resveratrol, a potent inhibitor of histone deacetylases, which activates Sirt1 and improves mitochondrial function, prevents diabetes-induced decrease in retinal Sirt1 and hyperacetylation of NF-*k*B [84]. In addition, epigallocatechin-3-gallate is considered a strong histone acetylase inhibitor, and shown to inhibit NF-*k*B activation [106]. In the development of diabetic retinopathy, hyperacetylation of NF-*k*B activates *MMP-9*, which is associated with retinal mitochondrial dysfunction and endothelial cell apoptosis [84]. Curcumin and genistein are also shown to regulate the epigenetic machinery [107]. Dnmt is activated in the *retina in diabetes, and activated Dnmt is shown to damage retinal mitochondria [70]. Dnmt inhibitors are now being in clinical trials for chronic diseases; FDA approved Dnmt inhibitor 5-azacytidine and 5-aza-20-deoxycitidine, are now in use for myeloid cancers and cutaneous T cell lymphoma [108]. With emerging data showing the importance of epigenetics in the development of diabetic retinopathy, prevention of such modifications could also prove an avenue to inhibit the development of retinopathy in diabetic patients.

12. Conclusion and perspective

Vision is the most indispensable means of communication, and diabetic retinopathy remains the leading cause of robbing a young patient of this valuable mean. The etiology of this slow progressing disease is very complex as number of metabolic, physiological and functional abnormalities have been identified playing important role in its pathogenesis. Despite extensive research in the field, maintaining tight glycemic control still remains the most viable option for preventing the development/progression of diabetic retinopathy, but tight glycemic control comes with added burdens of possible hypoglycemia and weight gain. Diabetes initiates a number of metabolic abnormalities in the retina, and oxidative stress is increased, which itself can also feed into these metabolic abnormalities. Mitochondria are damaged with a suboptimal electron transport chain, and damage to mtDNA further compromises the electron transport chain. To make the bad system worse, scavenging of free radicals and repair of the damaged mtDNA are also compromised, and free radicals continue to accumulate. Genes encoding
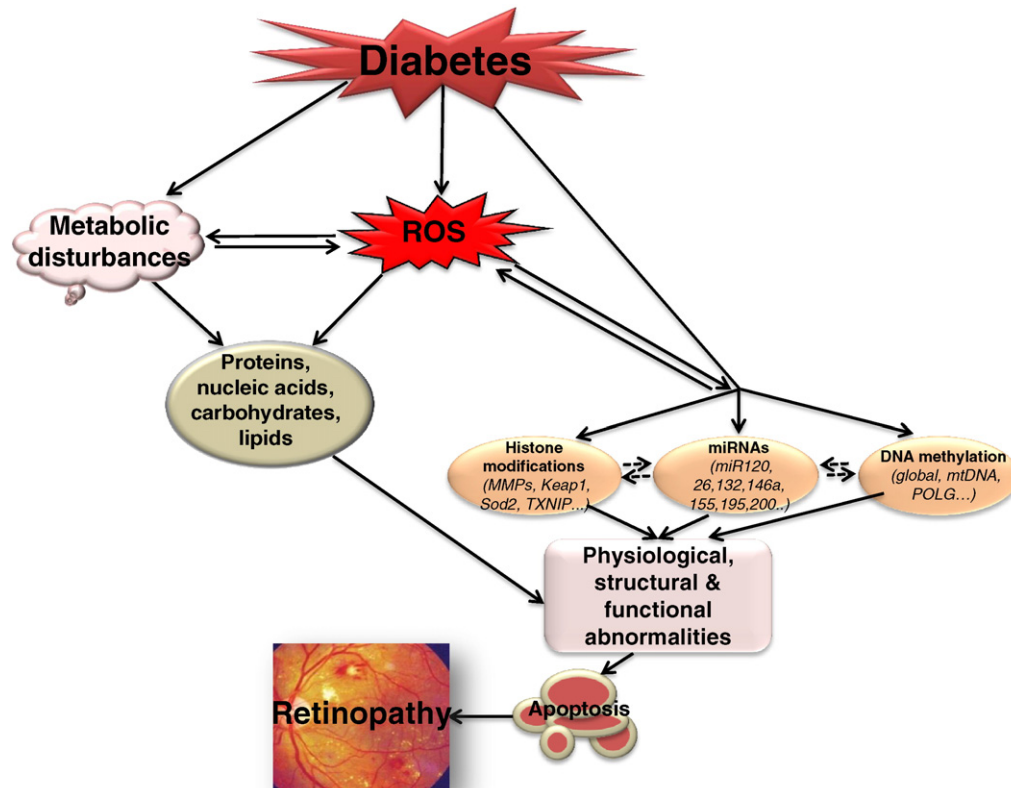
Fig. 3. Schematic representation shows diabetes-induced metabolic disturbances, reactive oxygen species (ROS), and epigenetic modifications. Increase in ROS degrades biomolecules (proteins, nucleic acid, lipids and carbohydrate), and epigenetic modifications (*via* histone modifications and DNA methylation) and miRNAs alter gene expression of proteins associated with maintain a redox balance. Capillary cell apoptosis is accelerated resulting in the development of diabetic retinopathy.

the proteins implicated in damaging the mitochondria (*MMP-9*) and in the antioxidant defense system (*Sod2*, *Keap-1 etc.*) are also epigenetically modified, further adding burden to the self-propagating vicious cycle of free radicals. Fig. 3 depicts the interrelationship between oxidative stress, metabolic disturbances and epigenetic modifications. Although experimental data with the use of antioxidants have been encouraging in preventing/slowing diabetic retinopathy, with the overwhelming data demonstrating the role of oxidative stress in its development, well-controlled longitudinal trial with antioxidants is warranted. Antioxidants could target multiple steps of oxidative stress and mitochondrial damage, and should be a welcoming sign for diabetic patients in preventing this sight-threatening disease.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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