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Oxidative stress and epigenetic modifications in the pathogenesis of diabetic retinopathy

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Abstract

Diabetic retinopathy remains the major cause of blindness among working age adults. Although a number of metabolic abnormalities have been associated with its development, due to complex nature of this multi-factorial disease, a link between any specific abnormality and diabetic retinopathy remains largely speculative. Diabetes increases oxidative stress in the retina and its capillary cells, and overwhelming evidence suggests a bidirectional relationship between oxidative stress and other major metabolic abnormalities implicated in the development of diabetic retinopathy. Due to increased production of cytosolic reactive oxygen species, mitochondrial membranes are damaged and their membrane potentials are impaired, and complex III of the electron transport system is compromised. Suboptimal enzymatic and nonenzymatic antioxidant defense system further aids in the accumulation of free radicals. As the duration of the disease progresses, mitochondrial DNA (mtDNA) is damaged and the DNA repair system is compromised, and due to impaired transcription of mtDNA-encoded proteins, the integrity of the electron transport system is encumbered. Due to decreased mtDNA biogenesis and impaired transcription, superoxide accumulation is further increased, and the vicious cycle of free radicals continues to self-propagate. Diabetic milieu also alters enzymes responsible for DNA and histone modifications, and various genes important for mitochondrial homeostasis, including mitochondrial biosynthesis, damage and antioxidant defense, undergo epigenetic modifications. Although antioxidant administration in animal models has yielded encouraging results in preventing diabetic retinopathy, controlled longitudinal human studies remain to be conducted. Furthermore, the role of epigenetic in mitochondrial homeostasis suggests that regulation of such modifications also has potential to inhibit/retard the development of diabetic retinopathy.

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Contributions

RAK and AK prepared and edited the review article, MM and BK contributed to writing portions of this article, and MM also prepared the figures.

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Keywords

Diabetic retinopathy; Epigenetic modifications; Mitochondria; Oxidative stress; Reactive oxygen species; Transcriptional regulation

1. Introduction

Diabetes is a major public health problem, and is now being considered as an epidemic of the 21st century. According to the numbers released by the International Diabetes Federation, in 2014 about 8.3% world's population (387 million people) have diabetes, and this number is expected to rise to over 590 million in less than 25 years. This is a life-long disease which affects function of multiple organs, leading to reduced quality of life, and, in some cases, to death (Whiting et al., 2011). Though high circulating glucose is the main hallmark of the disease, hyperglycemia could be either due to destruction of insulin producing beta cells, resulting in insulin deficiency (type I diabetes), or due to insulin resistance, which is generally followed by decreased insulin secretion and glucose intolerance (type II diabetes) (Unger, 2008). The disease carries a heavy social and economic burden; as per the International Diabetes Federation, in 2014, ~4 million deaths (9% of the global total) were associated with diabetes, and overall, direct health care costs of diabetes ranged from 2.5% to 15% annual health care budgets, depending on local diabetes prevalence and the sophistication of the treatment available. Despite significant improvement in education, advancement in technology and strategies, the prevalence of diabetes continues to increase and the need to protect the patients from its complications remains very high.

Chronic elevation in circulating blood glucose damages blood vessels, which results in many micro- and macro-vascular complications. Diabetic retinopathy is one of the major micro vascular complications affecting the vision, and is the leading cause of blindness in working age adults. With recent progress in the management of diabetes, fortunately the macro-vascular mortality is declining, but, diabetic patients are living longer and the incidence of retinopathy and loss of vision associated with this is increasing. Worldwide, approximately 93 million people have diabetic retinopathy, which includes 17 million with proliferative diabetic retinopathy and 28 million with sight-threatening diabetic retinopathy. As the incidence of diabetes is increasing at an alarming rate, in 2030, ~155 million of diabetic patients are projected to have retinopathy, with more than 51 million among them facing vision-threatening retinopathy (Whiting et al., 2011), and early detection and treatment remain the gold standards for its management.

2. Development of diabetic retinopathy

Diabetic retinopathy is a slow progressing chronic disease, at first patients do not show any symptoms, but, when their vision begins to impair, the chances are that retinopathy is already in its advanced stage, and if not controlled, could result in vision loss (Frank, 2004). The earliest clinical signs are mainly microaneurysms and intraretinal hemorrhages, and over 95% of patients with 20 years of diabetes and 80% patients with type II diabetes, show these signs (Aiello et al., 1998; Frank, 2004). With the progression of the disease, the

number and size of hemorrhages increase and occlusion of precapillary arterioles results in nerve fiber layer infractions, and cotton-wool spots start to appear. Eventually, due to nonperfusion of capillaries, new blood vessels begin to grow, leading to proliferative diabetic retinopathy, and, if left untreated, to retinal detachment (Frank, 2004) (Fig. 1). Animal studies have shown that in the early stages of diabetic retinopathy, the basement membrane thickens and capillary cells undergo accelerated apoptosis, resulting in degenerative capillaries and pericyte ghosts (Kern et al., 2000; Kowluru and Odenbach, 2004a; Robinson et al., 2012).

Retinal cells, other than vascular cells, including ganglion cells, also degenerate before vascular cells degeneration (Kern, 2014), and changes in retinal function (electroretinograms and contrast sensitivity) can be seen in patients before any abnormalities in their vasculature (Christ et al., 2014; Kizawa et al., 2006). Another important change that can occur as diabetic retinopathy progresses is diabetic macular edema; the blood-retinal barrier breaks down allowing leakage of plasma from small blood vessels into the macula, resulting in the swelling of the central retina and loss of central vision; these topics are reviewed by experts in the field (Antonetti et al., 2012; Bandello et al., 2010), and are beyond the scope of this article.

2.1. Risk factors

2.1.1. Hyperglycemia—Hyperglycemia remains the major instigator of the development of diabetic complications, including retinopathy. Due to sustained hyperglycemia, cellular metabolism is altered and the macromolecules undergo stable modifications. These acute and cumulative alterations in the cellular metabolism and macromolecules result in structural and functional changes in the tissue. The landmark Diabetes Control and Complications Trial (DCCT) has demonstrated that intensive glycemic control reduces the risk of development and progression of retinopathy by ~76% compared to the patients with conventional glucose control, and similar conclusions are also reported by the UK Perspective Diabetes study Group (Diabetes Control and Complications Trial Research Group, 1993; UKPDS, 1998).

2.1.2. Systemic factors—Cross-sectional and longitudinal studies have clearly documented that, in addition to sustained hyperglycemia, hypertension and dyslipidemia are also important factors in the development of diabetic retinopathy (Yau et al., 2012). Increased levels of oxidized lowdensity lipoprotein are associated with increased risk for progression to advanced retinopathy in patients with type I diabetes (Lyons et al., 2004). The Fenofibrate Intervention and Event Lowering in Diabetes study has revealed that the regulation of triglycerides by fenofibrates in type II diabetic patients reduces the number of laser intervention of proliferative diabetic retinopathy (Chew et al., 2014; Keech et al., 2007). In addition, the Action to Control Cardiovascular Risk in Diabetes Eye study has documented significant reduction in the progression of pre-existing diabetic retinopathy by fenofibrates and simvastatin (Chew et al., 2014). Experimental models (*in vitro* and animal models) have shown that saturated free fatty acids induce apoptosis of retinal microvascular cells, and administration of a docosahexaenoic acid-rich diet to type II diabetic animals prevents retinal inflammation and vascular pathology (Chen et al., 2005; Fu et al., 2014).

Moreover, blood pressure control in type II diabetic patients with hypertension is associated with inhibition of the progression of diabetic retinopathy (Chew et al., 2014). Thus, these systemic factors also appear to play important role in the development and progression of diabetic retinopathy (Fig. 2).

2.1.3. Genetic factors—In addition to metabolic and physiologic factors, pathogenesis of a disease is also influenced by genetic factors. The risk of severe diabetic retinopathy is about 3-fold higher in siblings of affected individuals, but the severity of retinopathy among diabetic patients with similar risk factors can show a varied range (Arar et al., 2008; Looker et al., 2007). Genome-wide association studies (GWAS) have identified a number of genetic variants that could explain some of the inter-individual variations in the susceptibility of diabetes. Significant variation in the *AKR1B1* gene, a gene encoding aldo-keto reductase family 1 member B1 (the rate limiting enzyme of the polyol pathway) is strongly associated with diabetic retinopathy (Abhary et al., 2009). The Wisconsin Epidemiologic Study of Diabetic Retinopathy has shown an association between a new potential single nucleotide polymorphisms *rs4865047* located in the *CEP125* gene and the severity of diabetic retinopathy (Grassi et al., 2012). However, single nucleotide polymorphisms (*rs6921438* and *rs10738760*) in vascular endothelial growth factor (VEGF)-related gene have not shown any association with the risk of retinopathy and nephropathy in diabetic patients (Bonnefond et al., 2013). Thus, although there could be added genetic contribution in the development and progression of diabetic retinopathy, the identification of susceptible loci through candidate gene approaches, linkage studies and GWAS remain in their early stages and this could be due to lack of power and/or lack of phenotype standardization.

2.1.4. Environmental factors—The incidence of diabetes is increasing at an alarming rate in developing countries, possibly due to changes in the lifestyle i.e. obesity, physical activity, diet and stress, and the prediction for future is alarming. Increased sugar consumption and processed food are some of the major factors in rapidly rising rates of obesity and type II diabetes, and are closely associated with the retinal abnormalities seen in overweight type II diabetic individuals (Kearney et al., 2014). Furthermore, increased VEGF, which is intimately associated with the development of diabetic retinopathy, is observed in the serum of obese patients (Miyazawa-Hoshimoto et al., 2003). Thus, environment also appears to play a major role in the development of diabetes and its complications.

2.2. Biochemical abnormalities induced by hyperglycemia

Clinical and experimental data have clearly indicated a very strong relationship between hyperglycemia and diabetic retinopathy. Sustained circulating high glucose can induce cellular dysfunction by generating toxic and reactive metabolites and also by interfering with a number of glucose-mediated signaling pathways (Brownlee, 2005; Giacco and Brownlee, 2010). Cutting edge research in the field has identified a number of hyperglycemia-induced cellular mechanisms that could account for the development of diabetic complications; some of the major metabolic abnormalities implicated in diabetic retinopathy include activation of protein kinase C (PKC), accumulation of polyols and advanced glycation end-products (AGEs), poly ADP-ribose polymerase (PARP), and increased flux through the hexosamine

pathway (Brownlee, 2005; Du et al., 2000; Kowluru and Kowluru, 2014; Kowluru, 2001, 2005b; Kowluru et al., 2013; Madsen-Bouterse and Kowluru, 2008; Mohammad and Kowluru, 2011b; Santos et al., 2011a).

Due to high circulating glucose in diabetic environment, the excess glucose is converted to sorbitol by aldose reductase, which is further oxidized to fructose by sorbitol dehydrogenase (Kador et al., 1990). While aldose reductase oxidizes NADPH to $NADP^+$, sorbitol dehydrogenase uses NAD^+ to form NADH; thus the active polyol pathway decreases NADPH and NAD^+ levels, the cofactors that are important in redox reactions. Increased polyol pathway activity is observed in the retina from animal models of diabetic retinopathy and from diabetic human donors with retinopathy (Dagher et al., 2004; Vinores et al., 1988). The role of polyol pathway in diabetic retinopathy is further strengthened by genetic association: the C allele of the polymorphism at position −106 in the promoter of aldose reductase gene *AKR1B1* is shown to be associated with diabetic retinopathy (Katakami et al., 2011). However, clinical trials using inhibitors of polyol pathway have failed to produce conclusive results (Sorbinil Retinopathy Trial Research Group, 1990), thus, undermining their use.

Diabetic environment also increases diacylglycerol levels in the retina and its capillary cells, which activates PKC (Xia et al., 1994). Activated PKC-δ can accelerate apoptosis of capillary cells and result in the formation of degenerative capillaries and pericyte ghosts (Geraldes et al., 2009), some of the early histopathological signs seen in animal models of diabetic retinopathy (Mizutani et al., 1996). In addition, activated PKC-δ can also increase redox-sensitive nuclear transcriptional factor, NF-*k*B, and decrease the survival signaling pathway of platelet-derived growth factor (Geraldes et al., 2009). Activation of PKC-β alters vascular endothelial cell permeability and blood flow (Aiello et al., 1997). Although RBX, an isoform-selective inhibitor of PKC-β, has produced beneficial effect in animal models of diabetic retinopathy, and ameliorated retinal hemodynamic abnormalities in diabetic patients, clinical trials using PKC inhibitors have not yielded very promising results (Davis et al., 2009), further demanding a need for additional clinical trials to prevent progression of this blinding disease.

Glucose, via nonenzymatic reactions, interacts with proteins or lipids and form Schiff's base and Amadori products, and their interactions subsequently form AGEs (Stitt, 2003). AGEs are increased in the retinal microvasculature from diabetic patients, and in serum AGEs are shown to correlate with the severity of retinopathy (Aso et al., 2000). AGEs also interact with cell-surface AGE-binding receptors (RAGEs), and this results in their degradation or cellular activation. Soluble RAGEs levels and polymorphisms in the gene encoding RAGE is considered to serve as a surrogate marker for patients vulnerable to diabetic complications (Ramasamy et al., 2011).

Diabetes also increases the flux of fructose 6-phosphate into the hexosamine pathway, and activation of this pathway serves as an alternative to glycolysis to utilize overproduction of fructose 6-phosphate (Brownlee, 2005). In the pathogenesis of diabetic retinopathy, activation of hexosamine pathway is associated with the apoptosis of endothelial cells and neurons (Du et al., 2003).

Clinical and experimental evidence has clearly demonstrated that diabetes increases oxidative stress (Baynes, 1991; Brownlee, 2005; Kowluru and Kennedy, 2001). In addition to circulating high glucose itself, diabetic conditions favor the production of reactive oxygen species (ROS) by activation of many metabolic abnormalities-induced by high glucose (Brownlee, 2005). Retina is rich in polyunsaturated fatty acids with high glucose oxygen uptake, which makes it a suitable target for oxidative damage (SanGiovanni and Chew, 2005). In diabetes, oxidative stress is increased in the retina and its capillary cells, and is considered as one of the major metabolic abnormalities associated with the development of diabetic retinopathy (Kowluru, 2003; Kowluru et al., 1999, 2001). The major biochemical abnormalities altered by hyperglycemic milieu are considered to be closely associated with increase in oxidative stress (Brownlee, 2005; Kowluru, 2001). Despite extensive research in the field, a link between any specific abnormality and the development of diabetic retinopathy is largely speculative, and this has raised a strong possibility that no single metabolic abnormality could be the sole cause of its development.

3. Oxidative stress

The body continuously produces ROS as a natural byproduct of the normal metabolism of oxygen, and mitochondria utilize over 95% of the oxygen to produce ATP, but, at the same time, mitochondria are also the major endogenous source of ROS (Turrens, 2003). Oxidative stress is a general term which mainly describes the steady state level of oxidative damage in a cell, tissue, or organ, caused by ROS. In the cytosol, ROS are also generated by nicotinamide adenine dinucleotide phosphate oxidase (Nox) (Frey et al., 2009). Although in normal conditions, ROS act as cellular messengers in redox signaling, increased ROS disrupt normal mechanisms of cellular signaling. If excess free radicals are not readily neutralized by antioxidants, they react with DNA base pairs altering gene expression, or cross-link with proteins or lipids (Cutler, 2005).

Antioxidants are considered as the first lines of defense to keep free radicals in check and prevent them from causing damage on other cells (Giugliano et al., 1996). The body is equipped with an efficient antioxidant defense system, which includes both enzymatic and nonenzymatic antioxidants (Valko et al., 2007). In many pathological conditions, e.g., as in diabetes, in addition to increased production of free radicals, antioxidant defense system is also compromised, which impairs the scavenging of these toxic radicals (Kern et al., 1994; Kowluru et al., 1994, 1997), and this increases oxidative burden (Fig. 3).

3.1. Major sources of free radicals

3.1.1. Cytosolic ROS—NADPH oxidase (Nox) serves as a major source of cytosolic ROS (Frey et al., 2009); NADPH act as electron donor and oxygen as electron acceptor, Nox2 catalyzes the one-electron reduction of oxygen to superoxide anion via oxidizing cytosolic NADPH to NADP (Bokoch and Zhao, 2006). Nox2 is a multiprotein enzyme comprising of cytosolic subunits-p47^{phox}, p67^{phox} and a small molecular G-protein Rac1, and the membrane-bound subunits p22^{phox} and the major catalytic subunit, gp91phox (Bokoch and Zhao, 2006; Kowluru et al, 2014a). Its activity is increased in diabetes in many

tissues, including pancreatic beta cells and retina (Kowluru and Kowluru, 2014; Kowluru et al., 2014a).

3.1.2. Mitochondrial ROS—The mitochondrial electron transport chain continuously reduces molecular oxygen to water. But, a small quantity of free radicals are released from complexes I, II and III resulting in incompletely reduced forms of oxygen, and due to increased release of electrons to the respiratory chain, mitochondrial membrane potential increases (Fulda et al., 2010). Most of these free radicals are transformed into more stable H_2O_2 by superoxide scavenging enzymes, but a small amount can leak out, and in pathological conditions with compromised scavenging enzymes, the levels of mitochondrial ROS increase further (Young et al., 2002). In addition, increased cytosolic ROS can also augment ROS by continuously damaging the mitochondria and increasing the membrane potential. Although complex III is the major source of ROS in the mitochondria, acyl-CoA dehydrogenase and glycerol phosphate dehydrogenase also generate ROS (Kowaltowski et al., 2009). Due to increased circulating glucose in diabetes, oxidation of glucose in the tricarboxylic acid cycle, electron donors are increased, which increases the voltage gradient across the mitochondrial membrane, and the transfer of electrons inside complex III is blocked (Brownlee, 2005).

4. Oxidative stress and diabetic retinopathy

It is well established that diabetes increases oxidative stress, and increased oxidative stress is observed in the retina and its capillary cells. As listed above, many of the biochemical abnormalities, experienced by the retina in diabetes, also have potential to increase oxidative stress (Kowluru, 2005a,b; Kowluru et al., 1999; Kowluru and Kanwar, 2009; Kowluru et al., 1997, 2014a).

4.1. Oxidative stress and metabolic abnormalities associated with diabetic retinopathy

Oxidative stress plays a major role in accumulation of AGEs by causing formation of reactive carbonyl compounds, which react with protein to form AGEs (Stitt, 2010). Moreover binding of AGEs to their receptors reduces intracellular antioxidant-glutathione (GSH), and depletion of GSH leads to reduced glyoxalase-1 recycling, which is important in reducing the cellular load of AGEs (Thornalley, 1998). AGEs can modify macromolecules and increase nitrative stress, and nitration can inactivate mitochondrial and cytosolic proteins and functions, and increase apoptosis, ultimately leading to pathological consequences (Kowluru et al., 2007; Liu et al., 2004). Increased oxidative stress also contributes to the formation of AGEs, and AGEs can increase oxidative stress as ROS are also the by-products of AGEs formation. Thus, AGEs and oxidative stress can feed into each other. DCCT, and follow up Epidemiology of Diabetes Interventions and Complications (EDIC) studies, have demonstrated a correlation between AGEs and the progression of diabetic retinopathy, and even 17 years into the EDIC study, skin collagen AGEs have shown a strong association with the future development of diabetic retinopathy (Genuth et al., 2015). Furthermore, by crosslinking with macromolecules, AGEs form intra-molecular and inter-molecular cross-links, and this increases nitrative stress, ultimately leading to capillary cell apoptosis in the retinal vasculature (Liu et al., 2004). AGEs also alter a number of other metabolic pathways

and functional abnormalities associated with diabetic retinopathy, including NF-*k*B signaling, and inner blood-retinal barrier breakdown (Moore et al., 2003).

Increased lipid peroxidation in diabetes also generates reactive aldehydes, such as 4-hydroxynonenal, malondialdehyde and acrolein, and these aldehydes further react with proteins generating relatively stable advanced lipoxidation end-products (ALEs). ALEs are considered to play a role in the pathogenesis of diabetic retinopathy-related microvascular dysfunction, possibly via impairing normal retinal Müller glia function (Curtis et al., 2011).

In response to the decreasing levels of NADPH by activated polyol pathway, the activity of hexose monophosphate shunt is increased, which, by increasing the ratio of NADH/NAD+, results in pseudohypoxia. Furthermore, fructose produced by the polyol pathway can be phosphorylated to fructose-3-phosphate, and, via serving as a glycosylating agents, this augments AGEs formation (Lorenzi, 2007; Szwergold et al., 1990). Activation of PKC, via increased biosynthesis of diacylglycerol, is also associated with ROS-mediated inhibition of glyceraldehyde 3 phosphate dehydrogeanse (GAPDH), a glycolytic enzyme which regulates the diacylglycerol precursor-triose phosphate (Brownlee, 2005). In addition, PKC itself is also activated by free radicals, and its inhibition is shown to reduce free radicals in hyperglycemic milieu (Meier and King, 2000). Hexosamine pathway is activated as an alternative to glycolysis for the utilization of hyperglycemia-induced overproduction of fructose-6-phosphate, and its activation is associated with endothelial cell and retinal neuron apoptosis (Nakamura et al., 2001; Xue et al., 2008).

Recent work has shown that diabetes also increases arginase activity in the retina, which, by decreasing arginine supply for nitric oxide synthase, reduces nitric oxide levels and increases superoxide generation (Narayanan et al., 2013). Superoxide reacts with nitric oxide, generating peroxynitrite, increased retinal peroxynitrite level are implicated in vascular and neural damage associated with diabetic retinopathy (Kowluru et al., 2007; Narayanan et al., 2013).

Diabetic retinopathy also shares similarities with a low grade chronic inflammatory diseases, and the levels of cytokines in the retina, and leukostasis is increased (Kern, 2007). ROS are considered a strong stimulus for the release of the cytokines, for example, interleukin-lβ itself can trigger signaling cascades resulting in excessive ROS (Vassilakopoulos et al., 2003). Administration of antioxidants to diabetic rats is shown to inhibit increase in retinal pro-inflammatory cytokines (Kowluru and Odenbach, 2004b).

Thus, there is overwhelming evidence for a bidirectional relationship between diabetes-induced major metabolic abnormalities and oxidative stress, making it an integral part of the disease process (Fig. 4).

4.2. Role of mitochondria

Mitochondria are the major endogenous source of superoxide, peroxynitrite and hydroxyl radicals (Young et al., 2002). Diabetic milieu damages retinal mitochondria, they become swollen and their membrane potential is altered, superoxide radicals are elevated and respiration rate is impaired. Their membrane transport machinery is impaired, biogenesis

and copy numbers become subnormal, and their electron transport chain system is compromised (Kanwar et al., 2007; Kowluru, 2005b, 2013; Kowluru and Abbas, 2003; Kowluru et al., 2011; Mishra and Kowluru, 2014; Santos et al., 2011b, 2012; Trudeau et al., 2010; Zhong and Kowluru, 2011a). Damage to the electron transport chain system prevents effective transfer of electrons during oxidative phosphorylation, and generates excessive ROS, resulting in a positive feedback cycle where ROS produce oxidative stress that eventually yields more ROS. In addition, mitochondrial DNA (mtDNA) is damaged, and the transcription of mtDNA-encoded genes, critical for functioning of the electron transport chain, becomes subnormal. Thus a vicious cycle of free radicals continues to self-propagate (Kowluru, 2013; Madsen-Bouterse et al., 2010a; Santos et al., 2011a, 2011b).

As stated above, due to high circulating glucose, more glucose becomes available to be oxidized in the tricarboxylic acid cycle, and this allows more electron donors to pass through the electron transport chain. In diabetic retinopathy, complex III activity is decreased and animal models have shown that overexpression of mitochondrial superoxide dismutase prevents mitochondrial dysfunction and the development of diabetic retinopathy (Kanwar et al., 2007; Madsen-Bouterse et al., 2010a,b). Furthermore, major metabolic abnormalities, associated with diabetic complications, are considered to be activated by increased free radicals via inhibition of GAPDH (Brownlee, 2005; Du et al., 2003). Due to decrease in GAPDH activity, the levels of its upstream glycolytic metabolite glyceraldehyde-3-phosphate are increased, and glyceraldehyde-3-phosphate activates AGEs, PKC and hexosamine pathways via increasing methylglyoxal, diacylglycerol and fructose-6 phosphate levels, respectively. Inhibition of GAPDH also increases glucose, which can further fuel into polyol pathway and increases the consumption of NADPH. In diabetes, decreased GAPDH glycolytic activity, possibly due to its posttranslational modifications, is seen in the retina and its capillary cells, and by translocating from cytosol to the nucleus, the enzyme becomes apoptotic (Du et al., 2003; Kanwar and Kowluru, 2009; Madsen-Bouterse et al., 2010b). Thus, hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain is considered to activate the major pathways of hyperglycemic damage (Brownlee, 2005; Kanwar et al., 2007). Overexpression of mitochondrial superoxide scavenging enzyme, manganese superoxide dismutase (MnSOD), in addition to inhibiting mitochondrial damage and the development of diabetic retinopathy, also prevents inhibition of GAPDH, suggesting a strong interrelationship between GAPDH and ROS (Kanwar et al., 2007; Kowluru et al., 2006a, 2006b; Madsen-Bouterse et al., 2010c; Mohammad and Kowluru, 2010; Santos et al., 2011b; Zhong and Kowluru, 2013a).

4.3. Antioxidant defense system

Antioxidants keep free radicals in check, and the enzymatic antioxidants work in concert with nonenzymatic antioxidants. The levels of intracellular nonenzymatic antioxidant GSH are decreased in the retina in diabetes, and this decrease in GSH is accompanied by a concomitant increase in oxidized GSH (GSSG) (Kern et al., 1994; Kowluru et al., 1994, 1997). GSH can be regenerated from GSSG by glutathione redox cycle (Meister, 1988), but in diabetes, the enzymes responsible for glutathione redox cycle (glutathione peroxidase and glutathione reductase) and biosynthesis/degradation (gamma-glutamyltranspeptidase and glutamate cysteine ligase) are also compromised (Kowluru et al., 1994, 1997). The cell is

also equipped with a redox sensitive transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 regulates the transcription of the enzyme critical in GSH biosynthesis, glutamate cysteine ligase, by binding at the promoter region, the antioxidant response element 4 (*ARE4*) (Lewis et al., 2010). In the pathogenesis of diabetic retinopathy, the transcriptional activity of Nrf2 and transcripts of the catalytic subunit of glutamate cysteine ligase are decreased (Zhong et al., 2013), further adding oxidative burden on the retina (Fig. 5).

Superoxide radicals are the major oxidants produced from a variety of sources, and superoxide dismutase scavenges these free radicals. While copper-zinc superoxide dismutase (Cu–Zn SOD) is located in the cytosol, mitochondria are equipped with the MnSOD, and these two isoforms act as bulk scavengers of superoxide (Yokoyama et al., 2002). In diabetes, the activities of both Cu–Zn SOD and MnSOD are decreased in the retina and its capillary cells, and overexpression of MnSOD prevents the development of retinopathy in diabetic mice (Kanwar et al., 2007; Kowluru et al., 1997). Thus, diabetic environment creates double whammy in the retina as, in addition to producing more oxidants, it also compromises the antioxidant defense system.

5. Mitochondria

Mitochondria are the powerhouse of the cell, and use about 90% of the oxygen, but superoxide radicals are also generated by the electron transport system, and this makes mitochondria vulnerable to the damaging effect of free radicals (Fulda et al., 2010). Retinal mitochondria are dysfunctional in diabetes, they are swollen and their morphology and membrane potential are altered, and respiration rate is impaired. Dysfunctional mitochondria leak cytochrome c out into the cytosol, initiating the apoptotic process (Kanwar et al., 2007; Kowluru, 2005b; Kowluru and Abbas, 2003; Trudeau et al., 2010; Zhong and Kowluru, 2011a). Regulation of mitochondrial superoxide by overexpressing *Sod2*, the gene encoding MnSOD, in mice, protects from accelerated apoptosis of capillary cell and the histopathology characteristic of diabetic retinopathy, further confirming the role of mitochondria in diabetic retinopathy (Kanwar et al., 2007).

5.1. Mitochondrial DNA damage

As reviewed above, ROS can damage macromolecules, including DNA, and increased levels of oxidatively modified guanine bases (8-OHdG) are observed in the retina in diabetes (Madsen-Bouterse et al., 2010a). Mammalian mitochondria have a double stranded small DNA (~16 kbp), which encodes only 13 protein, and these proteins are critical for proper functioning of the electron transport chain and for normal mitochondrial homeostasis (Scarpulla, 2012). Mitochondrial DNA is circular and lacks protective histones (Chen and Butow, 2005), and compared to the nuclear DNA (nDNA), mtDNA is prone to more extensive and persistent damage (Stuart and Brown, 2006). Damaged mtDNA impairs transcription and protein synthesis, and this further compromises electron transport and exacerbates ROS production (Madsen-Bouterse et al., 2010a; Van Houten et al., 2005). In addition to increased 8-OHdG levels in the retinal mtDNA in diabetes, increased variants in mtDNA are also observed in diabetes (Madsen-Bouterse et al., 2010a; Mishra and Kowluru,

2014) (Fig. 6). However, mitochondria are also equipped with efficient DNA repair machineries, including base excision repair (BER) and mismatch repair (MMR) systems (Santos et al., 2013b). BER system is the primary repair pathway for oxidative damage, and it recognizes, excises and replaces small base modifications in the DNA (Santos et al., 2013b). MMR system repairs uncomplimentary base pairs incorporated into the DNA and the insertion and/or deletion loops formed during replication (Kazak et al., 2012). MMR pathway has a number of proteins to assist in detecting the mismatches and their repair, e.g., Msh2 of MutS family recognizes mismatches and MutL homolog 1 (Mlh1) of MutL cuts the mismatch (Kazak et al., 2012). While Msh2 is largely associated with nDNA polymerase beta, Mlh1 works with mtDNA polymerase gamma (POLG) (Martin et al., 2010). In addition to mtDNA damage, diabetes also compromises the repair machinery (Madsen-Bouterse et al., 2010b, 2010c), while the gene expression of BER enzymes are increased, their translocation to the mitochondria is decreased, and the major MMR proteins, Mlh1 and Msh2 are also decreased. Overexpression of *Mlh1* in retinal endothelial cells prevents glucose-induced damage to the mtDNA and reduces sequence mismatches, and also ameliorates their accelerated apoptosis (Mishra and Kowluru, 2014). Thus, diabetic environment, induces mtDNA damage, and also compromises the repair of the damaged DNA (Madsen-Bouterse et al., 2010a; Mishra and Kowluru, 2014), further compromising mitochondrial homeostasis.

Mitochondrial DNA has a large non-coding sequence, the displacement-loop (D-loop), which contains the essential transcription elements, and this highly vulnerable unwound region provides control sites for mtDNA replication (Clayton, 2000; Rothfuss et al., 2010). In diabetes, D-loop experiences more damage and sequence variants than other regions of mtDNA, and its copy numbers are decreased (Madsen-Bouterse et al., 2010c; Mishra and Kowluru, 2014; Santos and Kowluru, 2011; Tewari et al., 2012a). In addition, mtDNA biogenesis, which is under dual genetic control of nDNA and mtDNA, is also compromised (Santos and Kowluru, 2011; Santos et al., 2011b). The mitochondrial transcription factor (TFAM), a key activator of mitochondrial transcription which helps maintain and organize mitochondrial genome, fails to reach to the mitochondria to initiate mtDNA transcription and replication, and mitochondria copy number and mass are decreased in the retina. The major membrane transport system of the mitochondria is also impaired, and the binding of TFAM with the membrane transport proteins is decreased. Furthermore, the binding of TFAM at the D-loop is attenuated resulting in subnormal transcription and compromised stability (Santos and Kowluru, 2013; Santos et al., 2014, 2011b, 2012; Tewari et al., 2012a; Tewari et al., 2012b; Zhong and Kowluru, 2011a). These studies have clearly indicated that diabetes damages mtDNA and decreases mitochondria copy numbers, reduces transcription of mtDNA, and decreases its biogenesis and replications. However, regulation of mitochondrial superoxide levels by overexpression of *Sod2* ameliorates diabetes-induced damage to the mtDNA integrity, and prevents decrease in mtDNA biogenesis and replication, suggesting that in diabetic retinopathy, mitochondria homeostasis is under the control of superoxide radicals (Kanwar et al., 2007; Santos et al., 2011b; Tewari et al., 2012a).

5.2. Diabetes and mitochondrial damage

As stated above, although it is well established that in the pathogenesis of diabetic retinopathy retinal mitochondria are damaged, but how diabetic milieu damages the mitochondria remains elusive.

5.2.1. NADPH oxidase—Animal studies have demonstrated that in diabetes, even though ROS are increased early in the retina and its capillary cells, mitochondrial damage follows cellular ROS increase (Kowluru and Abbas, 2003; Santos et al., 2012). While mitochondria are the predominant source of ROS (Murphy, 2009), a significant amount is also generated via transferring electrons across the membrane from NADPH to molecular oxygen, and Nox2 is also activated in vascular cells of the retina (Kowluru et al., 2014a; Paravicini and Touyz, 2008). Increased ROS can damage mitochondrial membranes and directly open the mtK_{ATP} channels decreasing the mitochondrial membrane potential and uncoupling mitochondrial respiration (Daiber, 2010). Our studies have shown that Nox2 activation is an early event in the development of diabetic retinopathy, and the results have suggested that the cytosolic ROS progressively damage the mitochondria leading to their dysfunction (Kowluru and Kowluru, 2014; Kowluru et al., 2014a).

5.2.2. Matrix metalloproteinases—Another mechanism responsible for mitochondrial damage in diabetic retinopathy is via activation of gelatin matrix metalloproteinases (MMPs) (Kowluru et al., 2011; Mohammad and Kowluru, 2011a). Although MMPs are cytosolic enzymes, diabetic environment favors their translocation into the mitochondria. The molecular mechanism by which redox-sensitive MMPs (MMP-2 and MMP-9) accumulate in the retinal mitochondria appears to be via the regulation of chaperons Hsp70 and Hsp60 (Kowluru et al., 2011; Mohammad and Kowluru, 2011a). Once inside the mitochondria, MMPs damage the mitochondria and increase their permeability via breaking down connexin 43. This allows Bax to move into the mitochondria, and activate the pro-apoptotic machinery (Mohammad and Kowluru, 2011a). Mice with *MMP-9* gene silenced are protected from mitochondrial damage and the development of diabetic retinopathy (Kowluru et al., 2011). Although activation of cytosolic MMP-9 and MMP-2 in the retina is an early event, their accumulation in the mitochondria follows their activation, and despite increase in the mitochondrial MMPs, mitochondrial dysfunction and damage to mtDNA occur at much later stage (Santos et al., 2013a) (Fig. 7).

The literature cited above suggests that due to increased production of cytosolic ROS in diabetes, MMPs are activated in the retina. Activated MMPs begin to damage mitochondrial membrane, mitochondria become swollen, and their membrane potentials are impaired. Complex III, one the major sources of superoxide production, becomes suboptimal, and the superoxide scavenging system is compromised, resulting in accumulation of free radicals (Kanwar et al., 2007; Kowluru et al., 2014a, 2011). With time, mtDNA is damaged and the system to repair damaged mtDNA is compromised, and the transcription of mtDNA-encoded proteins that are critical in the integrity of the electron transport system is hampered (Madsen-Bouterse et al., 2010c, 2010a; Santos and Kowluru, 2011; Tewari et al., 2012b). Decreased mtDNA biogenesis and impaired transcription further augments superoxide accumulation, and this vicious cycle self-propagates (Fig. 8). Although during the initial

stages of the diabetic insult, the retina tries to overcome increased ROS by increasing the transcription of the genes important in mtDNA biogenesis and repair, but with sustained insult, this mechanism is also overwhelmed (Santos et al., 2012).

6. Epigenetics and gene regulation

The expressions of many proteins, including enzymes responsible for removing free radicals, are altered in the retina and its capillary cells in diabetes resulting in metabolic abnormalities (Madsen-Bouterse et al., 2010a; Mishra et al., 2014b; Santos et al., 2011a, 2013a; Zhong and Kowluru, 2011b, 2013b). In addition to the transcriptional factors, gene expression is also regulated by metabolite fluctuations, and the external environment's effects upon genes can influence disease. Epigenetics, covalent modifications of DNA, which does not alter its primary sequences, acts as interplay between genes and the environment. Although epigenetic is a natural phenomenon, and these regular changes are acquired throughout life, it can be influenced by environment, lifestyle and disease (Gemenetzi and Lotery, 2014; Regha et al., 2007). Unlike inherited genetic variations that are static through the life course, epigenetic changes are dynamic, and vary depending on the tissue and the disease state, but, once the changes are established, they can be relatively stable (Bishop and Ferguson, 2015). The major epigenetic changes are the methylation of DNA and posttranslational modifications in the histones. The epigenetic machinery responsible for altering the chromatin structure comprises of histone modifying enzymes, DNA modifying enzymes, chromatin remodeling proteins, non-coding regulatory small RNAs and histone variants (Gemenetzi and Lotery, 2014; Reddy et al., 2015; Regha et al., 2007).

6.1. DNA methylation

As DNA is not a static entity, it can modify its properties in response to pathological and environmental stimuli (Majumdar et al., 2011). Methylation of cytosine forms 5-methylated cytosine (5mC), which changes protein-DNA interactions leading to alterations in chromatin structure. Although the majority of the genome has low cytosine-guanine content, they also contain cytosine-guanine-rich regions, the CpG islands. These islands are usually enriched in the promoter regions, and cytosine in these islands is unmethylated (Deaton and Bird, 2011). Formation of 5mC in the promoter region blocks the binding of the transcriptional activators to the concurrent DNA sequences. DNA methylation is brought about by DNA methyltransferases (Dnmts), and these enzymes use S-adenosyl methionine (SAM) as the methyl donor (Deaton and Bird, 2011; Majumdar et al., 2011). There are three such enzymes in mammals: Dnmt1, Dnmt3a, and Dnmt3b. Although there is another isoform-Dnmt3L, it is catalytically inactive and serves as a cofactor for Dnmt3a and Dnmt3b, the two de-novo enzymes. Dnmt1 is a maintenance enzyme, and it regulates tissue-specific patterns of methylated cytosine residues (Deaton and Bird, 2011; Majumdar et al., 2011). This isoform has preferential activity for hemi-methylated DNA. Pathological Dnmt activity and aberrant 5mC formation have been linked with neurodegeneration (Chestnut et al., 2011). Oxidation of 5mC by ten-eleven translocation enzymes forms 5-hydroxymethylcytosine (5hmC), and 5hmC are susceptible to further oxidation to generate 5-formylcytosine and 5-carboxylcytosine (Zhang et al., 2013). Active DNA demethylation is promoted by a family of DNA glycosylases (TDG), which removes 5-methylcytosine base, followed by cleavage

of the DNA backbone at the abasic site, and the methylated cytosine is replaced by an unmethylated cytosine (Kohli and Zhang, 2013). In contrast, the passive process involves absence/inactivation of Dnmt1 resulting hypomethylated DNA (Zhu, 2009). Thus, the overall DNA methylation is a dynamic process.

6.2. Histone modifications

DNA is wrapped around nucleosomes, and the nucleosome is comprised of two copies of each histone: H2A, H2B, H3, and H4. The N-terminal tails of the histones are subject to posttranslational modifications, including methylation, acetylation, ubiquitination, sumoylation, and phosphorylation. These modifications are regulated by a balance between the enzymes inserting or removing a group (Sarkar et al., 2013). This insertion or removal can either open or restrict access to the DNA, influencing numerous biological processes including transcription, replication, and chromosome maintenance (Fig. 9). Acetylation of histones removes the positive charge, and opens up the chromatin for transcriptional activation by allowing recruitment and binding of the transcription factor and RNA polymerase II (Glozak and Seto, 2007). Acetylation status of histones is maintained by a group of enzymes with opposing functions; while histone acetyltransferases (HATs) inserts an acetyl group on a lysine of the histone, histone deacetylases (HDACs) remove the acetyl group (Sarkar et al., 2013).

Methylation, in most cases, turns the gene ‘off and demethylation ‘on’, the process is dependent on the target site (Kouzarides, 2002); e.g., while trimethylation of histone H3 at lysine 4 (H3K4me3) is generally considered as an active mark for transcription, dimethylation of histone H3 at lysine 9 (H3K9me2) as a transcriptional silencing mark. In addition, the degree of methylation at specific lysine residues (mono-, di- or tri-methylation) also determines the outcome of methylation; monomethylated H4K20 (H4K20mel) and H4K20me3 are considered as transcriptional repressors, H4K20me2 is largely considered as an activator (Kubicek et al., 2007; Nightingale et al., 2006). Methylation is tightly regulated by methyltransferases, but histone demethylation machinery is not yet well identified; a lysine-specific histone demethylase, LSD1, has been identified to specifically remove methyl group from monomethylated H3K4 and H3K9 (Musri et al., 2010).

6.3. MicroRNAs

Gene expression can also be regulated at post-transcriptional level by a class of non-coding RNAs, microRNAs (miRNAs). These single-stranded RNA molecules are approximately 22 nucleotides in length, and can bind to the target sites within the 3′UTR of the targeted mRNA. Gene silencing occurs via either mRNA degradation or prevention of its translation (Dykxhoorn et al., 2003; Garzon et al., 2009). Regulation of gene transcription by miRNA is somewhat complex; several miRNAs often work together to lower the expression of a shared target mRNA as individual mRNAs may contain multiple binding sites for different miRNAs, individual miRNA can target as many as 100 different mRNAs (Caroli et al., 2013).

Although both histone modifications and DNA methylation independently regulate gene transcription, recent studies have shown that DNA methylation and histone modifications

can also work in concordance (Cedar and Bergman, 2009); for example, gene transcription could also be regulated by association of Dnmt1, methyl CpG binding protein 2 (MeCP2) and methyl-CpG-binding domain proteins (MBDs) proteins with HDAC (Fahrner et al., 2002) or DNA methylation could cooperate with histone methyltransferase SETDB1/ESET in trimethylation of H3K9. Dnmt can also interact with histone modifying enzymes, SUV39h1 and EZH2 lysine histone methyltransferases to regulate their function (Rai et al., 2010; Wakabayashi et al., 2014), suggesting that the transcription of the same gene could be regulated by a possible cross talk between histone modifications and DNA methylation. In addition, miRNA can also regulate the enzymes responsible for DNA and histone modifications (Wakabayashi et al., 2014). Thus, there appears to be a close interplay between these different pathways of posttranslational modifications of gene regulation. Due to the reversible nature of the epigenetic modifications, their response to the changing environment and possibility of being passed on to successive generations makes them critical targets for therapeutic intervention in chronic diseases like diabetes and its complications.

7. Epigenetic modifications in diabetes

As epigenetic changes are influenced by environment, lifestyle and disease (Gemenetzi and Lotery, 2014; Regha et al., 2007), it is shown to play an important role in impaired glucose tolerance (Sterns et al., 2014). Recent studies have also revealed that cytokines and other metabolites can affect DNA methylation, and by acutely reprograming gene expression, could contribute to the type II diabetes (Raciti et al., 2014). In addition, acetyl H3K9 status of *HLA-DRB1* and *HLA-DQB1*, the genes highly associated with type I diabetes, is considered to play an important role in their transcriptional regulation (Miao et al., 2012), implying the role of epigenetic in diabetes.

The landmark DCCT studies have documented that hyperglycemia is the major instigator of diabetic complications, and the follow up EDIC studies have clearly shown that diabetic complications do not revert even after glycemic burden is abolished for a number of years (Aiello, 2014; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group, 2005). These clinical studies are also supported by experimental studies using *in vivo* and *in vitro* models; a metabolic memory phenomenon has been suggested in the continued progression of diabetic complications after termination of hyperglycemia (Engerman and Kern, 1987; Kowluru, 2003; Kowluru et al., 2004; Zhong and Kowluru, 2010, 2011b). Since epigenetic is non-genetic cellular memory, which can be modulated by environmental signals, and as mentioned above, sustained high glucose results in many metabolic, biochemical and genetic abnormalities, this makes epigenetic modifications as possible candidates for diabetic complications. In fact, diabetic environment is shown to facilitate epigenetic modifications in various organs associated with micro- and macrovascular complications (Kato and Natarajan, 2014; Kowluru et al., 2013; Stankov et al., 2013). Genome-wide DNA methylation study has identified 19 potential CpG sites in the blood cells from type 1 diabetic patients that undergo DNA methylation in diabetes (Bell et al., 2010). Deficiency of SAM is reported in the diabetic patients with nephropathy (Poirier et al., 2001), and increased global DNA methylation levels are observed in diabetic patients with albuminuria

compared with those in normal albumin level (Maghbooli et al., 2015, 2014). Monocytes from diabetic patients present a strong association between acetyl H3K9 and mean hemoglobin A1C levels during the DCCT and EDIC tenure (Miao et al., 2014). High glucose-induced upregulation of interferon-stimulated genes in human monocyte have higher acetyl H3K9 levels at high and low CpG sites of their promoters (Miao et al., 2013). Vascular smooth muscle cells derived from type II diabetic mice show irreversibly decreased levels of both H3K9me3 and methyltransferase Suv39Hl at the promoter regions of interleukin-6 and monocyte chemo-attractant protein1 (Villeneuve et al., 2008). Furthermore, in human monocytic THP-1 cells, aberrant changes in H3K4me2 and H3K9me2 are associated with increased histone acetylation in the chromatin region containing the promoter of the transcription factor, NF-*k*B (Miao et al., 2013).

7.1. Epigenetic modifications in diabetic retinopathy

Diabetic retinopathy is a multifactorial disease, and this slow progressing disease continues to progress after cessation of hyperglycemia, suggesting that epigenetics could be one of the major contributing participants in its development and progression, but the role of epigenetic in diabetic retinopathy is still in its incipient stages.

7.1.1. DNA methylation—DNA methylation is closely associated with the regulation of gene transcription, and in a case–controlled study of 168 patients with type II diabetes, global DNA methylation status is shown to be associated with retinopathy (Maghbooli et al., 2015). However, despite showing no association between the risk factors of diabetic retinopathy, e.g., hyperglycemia, dyslipidemia and hypertension, methylation status of DNA shows a strong correlation with the progression of retinopathy (Maghbooli et al., 2015, 2014). In addition, aberrant DNA hypermethylation of dimethyl arginine dimethylaminohydrolase 2, a protective factor for endothelial function, is linked to the dysfunction of endothelial progenitor cells (Niu et al., 2014). We have shown that the activity of Dnmt is increased in the retina and its capillary cells in diabetes, and the regulatory region of DNA polymerase gamma, an enzyme important in mitochondrial DNA biogenesis, is hypermethylated, resulting in the downregulation of its expression (Tewari et al., 2012b).

7.1.2. Histone modifications—In addition to altered DNA methylating machinery, histone modifying machinery is also affected in diabetes. An association between retinopathy and the polymorphism in the gene that encodes histone methyltransferases, *SUV39H2*, is observed in diabetic patients (Syreeni et al., 2011). Using *in vitro* and *in vivo* models of diabetic retinopathy, our group has shown increased HDACs and decreased HAT activities and global acetylation (Zhong and Kowluru, 2010), but, others have shown increased histone acetylation (Kadiyala et al., 2012), and the reason for such discrepancy is not clear. Gene specific epigenetic modifications have revealed that the promoter and enhancer regions of *Sod2* are altered; while H4K20me3, acetyl H3K9 and NF-*k*B p65 are increased, methylation of H3K4 is decreased in diabetes (Zhong and Kowluru, 2011b, 2013a). Decrease in H3K9me2 at retinal *MMP-9* promoter, with concomitant increase in acetyl H3K9, enables the binding of p65 subunit of NF-*k*B, resulting in its increased expression in diabetes (Zhong and Kowluru, 2013b). Furthermore, increased histone

methyltransferase Set7 recruitment at the promoter of NF-*k*B in hyperglycemic milieu is associated with its increased transcription (El-Osta et al., 2008), and activated NF-*k*B increases the apoptosis of retinal capillary cells in diabetes (Romeo et al., 2002). The function of Nrf2 is also epigenetically modified, impeding its transcriptional activity (Mishra et al., 2014b). Thioredoxin-interacting protein is implicated in promoting epigenetic alterations at the promoter of cyclooxygenase-2 via mitogen-activated protein kinase–NF–*k*B signaling pathway (Perrone et al., 2009). Thus, in diabetic environment, modifications in DNA and histones appear to modulate expressions of many genes important in the pathogenesis of diabetic retinopathy.

7.1.3. MicroRNAs—The levels of the small non-coding RNAs are also altered in diabetes; patients with proliferative and nonproliferative diabetic retinopathy have different serum levels of miR-21, miR-181c, and miR-1179 (Qing et al., 2014). Theses miRNAs are highly modulated by oxidative stress; especially, miR-200 family members are shown to play a critical role in oxidative-stress dependent endothelial dysfunction and diabetic retinopathy (McArthur et al., 2011). Experimental models have shown that downregulation of miR-126, miR-146a and miR-200b is associated with upregulation of *VEGF*, and that of miR-146a with, also with fibronectin production. Upregulation of miR-195 is shown to downregulate deacetylase Sirtuin 1 (Sirt1) (Chen et al., 2013), and in diabetic retinopathy, inhibition of Sirt1 in the retina, activates NF-kB, a redox-sensitive pro-apoptotic factor (Kowluru et al., 2014b). In contrast, upregulation of miR-29b is considered to be protective against apoptosis of the retinal ganglion cells in the early stages of diabetic retinopathy (Mastropasqua et al., 2014; Silva et al., 2011). Furthermore, miR-200b and histone acetylator p300 regulate TGFβ-mediated increased production of mesenchymal markers and reduced production of endothelial markers, induced by high glucose in endothelial cells (Cao et al., 2014). These studies have clearly suggested a role of miRNAs in regulating various aspects of diabetic retinopathy, including blood retinal breakdown and neovascularization.

7.2. Oxidative stress, epigenetics and mitochondrial homeostasis

As mentioned above, oxidative stress plays an important role in the pathogenesis of diabetic retinopathy, and increased ROS have detrimental effects on cellular components, including proteins, lipids and DNA (Valko et al., 2007). The majority of enzymes responsible for maintaining methylated status of DNA and methylated/acetylated status of histones are redox-sensitive in nature. For example, Dnmts are redox-sensitive enzymes (Ziech et al., 2011), and superoxide can also enhance DNA methylation via deprotonating the cytosine molecule and accelerating the reaction of DNA with the SAM (Afanas'ev, 2014). Oxidative damage to DNA results in epigenetic changes in chromatin organization by inhibiting the binding of the methyl-CpG binding domain of MeCP2 (Valinluck et al., 2004), and disruption of epigenetic mechanisms in oxidative stress (Chervona and Costa, 2012). Oxidation of cysteine residues in HDAC eases the release of deacetylase from the nucleus, which can be prevented by thioredoxin1 (Matsushima et al., 2013). Histone demethylase LSD1 is a FAD-dependent amine oxidases, which requires oxygen to function (Wang et al., 2012). Oxidative stress is also shown to inhibit the activity of HDACs, and increase acetylation in miR-466h-5p (Druz et al., 2012).

Retina from human donors with documented diabetic retinopathy have shown increased acetyl H3K9 at *MMP-9* promoter, which enables the recruitment of p65, and as mentioned above, activated retinal MMP-9, via damaging the mitochondria initiates the apoptotic machinery (Kowluru, 2010; Kowluru et al., 2011). Sirt1 regulates the acetylation status of p65 subunit, and the activity of Sirt1 is decreased in the retina in diabetes (Kowluru et al., 2014b) (Fig. 10). Histones are also modified at the promoter and enhancer of *Sod2*, and the binding of LSD1 and NF-*k*B are increased, resulting in downregulation of *Sod2* (Zhong and Kowluru, 2011b, 2013a). In addition, epigenetic modifications at the promoter of Kelch-like ECH associated protein 1 (*Keap1*, an intracellular inhibitor of Nrf2), increase the binding of transcriptional factor Sp1 and the *Keap1* is upregulated. Keap1 tries to restrain Nrf2 in the cytosol, which impairs the transcriptional activity of Nrf2, and the cytoprotective proteins are downregulated. Due to increased H3K4me1, the binding of Nrf2 at its glutamate-cysteine ligase-ARE4 (*Gclc-ARE4)* is decreased, the catalytic subunit of glutamate-cysteine ligase is downregulated, and the biosynthesis of GSH is attenuated (Mishra et al., 2014a,b; Zhong et al., 2013) (Fig. 11). Diabetes-induced epigenetic modifications appear to produce double whammy-activation of MMP-9 damages mitochondria and increases oxidative stress, and downregulation of *Sod2* and that of glutamate-cysteine ligase decrease the enzymatic and non-enezymatic antioxidant defenses respectively. Thus, it is becoming apparent that the diabetic environment favors epigenetic modifications in the retina; DNA methylation and miRNAs are altered and histones are modified. Due to epigenetic modifications, the binding of transcription factors (e.g., NF-*k*B, Sp1 and Nrf2) and the expression of genes (e.g., *Sod2*, *MMP-9, Keap1, TXNIP, VEGF)* become abnormal, resulting in the metabolic, physiological and structural abnormalities, and eventually, in the development of diabetic retinopathy.

8. Epigenetics and mitochondrial homeostasis

8.1. Methylation of mtDNA

Mitochondrial homeostasis is maintained by a close cooperation between nDNA and mtDNA as this small DNA, which lacks histones. Rest of the proteins required for its biosynthesis, structural stability and function are synthesized by the nDNA (Scarpulla, 2012). Epigenetic changes in nDNA are now emerging as important areas of investigation in many acquired chronic diseases, but, epigenetic changes in mtDNA is still in its early stages, and due to lack of histone complexes in mtDNA, the focus has been on the DNA methylation in mtDNA stability and mitochondrial function. Although mtDNA represents less than 1% of the total cellular DNA, it has ~450 CpG sites and ~4500 cytosines at non-CpG sites (Branco et al., 2011). Formation of 5mC and 5-hydroxymethylcytosine in mtDNA is shown to be associated with chronic diseases, including cancer (Dzitoyeva et al., 2012; Iacobazzi et al., 2013; Manev et al., 2012), and DNA methylation machinery has been identified in the mitochondria (Shock et al., 2011). Methylation of mtDNA decreases with age, and hypo methylation is linked with the decreased transcriptional capacity of proteins encoded by mtDNA; for example, increase in Dnmt1 in the mitochondria is shown to up-regulate mtDNA-encoded NADH dehydrogenase 1 and down-regulate *Cytochrome b* (Dzitoyeva et al., 2012; Shock et al., 2011). Our recent studies have shown that Dnmt expression is increased in the retinal mitochondria in diabetes, and mtDNA is hyper-methylated with increased 5mC levels. The D-loop region experiences more damage and has

higher methylation than other region of the mtDNA, and inhibition of *Dnmt1* by its si-RNA in retinal endothelial cells prevents hyperglycemia-induced mitochondrial damage and restores mtDNA transcription. These results have clearly suggested a critical role of mtDNA methylation in the development of diabetic retinopathy (Fig. 12).

8.2. Mitochondrial miRNAs

Mitochondrial function is also regulated by miRNAs, and this regulation can be achieved either by targeting nDNA- or mtDNA-encoded genes (Duarte et al., 2014). For example, miR-126 is shown to regulate mitochondrial function and metabolism, possibly by inhibiting the Akt pathway, as it restores the tricarboxylic acid cycle for the synthesis of ATP (Tomasetti et al., 2014). Recently some miRNAs are also found in the mitochondria, though the source of their transcription (nDNA or, potentially, the mtDNA) remains unclear. Human mtDNA is shown to have mitomiR sequences, namely, miR-1974, miR-1977 and miR-1978, but these mitomiRs, compared with cytosolic miRNAs, lack preferential targeting of nDNA-encoded mitochondrial genes (Kren et al., 2009). How diabetes affects these mitomiRs, and their role in maintaining mitochondrial homeostasis in diabetic retinopathy remains to be investigated.

In summary, epigenetics is an emerging area, and the information provided above suggests that the diabetic environment favors epigenetic modifications; the genes implicated in the pathogenesis of diabetic retinopathy and enzymes responsible for epigenetic modifications and miRNAs are altered in the retina in diabetes, suggesting the role of these posttranslational modifications in the development of diabetic retinopathy (Fig. 13).

9. Therapeutic implications

9.1. Antioxidant defense system

The literature reviewed here clearly suggests that oxidative stress has a major role in the etiology of diabetic complications, including retinopathy, and mitochondrial damage is an important component as the damage to the mtDNA, via damaging the electron transport system, fuels into a vicious cycle of superoxide (Brownlee, 2005; Kowluru et al., 2013).

9.1.1. Animal studies—Experimental data have demonstrated that the regulation of oxidative stress by antioxidants and maintenance of mitochondrial homeostasis prevents/retards the development of diabetic retinopathy. Administration of antioxidants (vitamins C and E, or multi-antioxidants mixture containing vitamins C and E, trolox, α-tocopherol, N-acetyl cysteine, β-carotene and selenium), soon after induction of diabetes in rats, attenuates the formation of acellular capillaries and pericyte ghosts, the early sign characteristic of diabetic retinopathy (Kowluru et al., 1996, 2001). Long-term administration of the antioxidants used in the Age-Related Eye Disease Study, the same nutritional antioxidants that have been demonstrated to slow the progression of age-related macular degeneration in patients, prevents the development of diabetic retinopathy (Kowluru et al., 2008a), and that of green tea, rich in polyphenolic compounds with potent antioxidant activity, ameliorates retinal histopathological abnormalities associated with diabetic retinopathy (Mustata et al., 2005). Supplementation with lipoic acid, a natural thiol antioxidant, or with carotenoid rich

diet, which is in clinical trials for 'Diabetes Vision Function', inhibits retinal capillary cell apoptosis and the development of retinopathy in diabetic rats (Kowluru and Odenbach, 2004a; Kowluru et al., 2014c). Benfotiamine, a fat-soluble synthetic derivative of vitamin B1, is shown to attenuate the severity of diabetic retinopathy in rodents, possibly by inhibiting the major metabolic pathways involved in the pathogenesis of diabetic retinopathy (Hammes et al., 2003). Zeaxanthin supplementation significantly inhibits oxidative damage and protects the retina from inflammatory mediators associated with the pathogenesis of diabetic retinopathy (Kowluru et al., 2008b), and lutein administration decreases the total retinal thickness and protect impairment in visual function (Arnal et al., 2009). Curcumin, a polyphenol with potent antioxidant and anti-inflammatory effects, ameliorates retinal oxidative stress and proinflammatory markers in diabetic rats (Kowluru and Kanwar, 2007). Treatment with FP15, a potent peroxynitrite decomposition compound, decreases diabetes-induced increased leukocyte entrapment in the retinal microcirculation (Sugawara et al., 2004), and PJ-34, a PARP inhibitor, prevents the early lesions of diabetic retinopathy (Zheng et al., 2004). Furthermore, inhibition of Tiam1-Rac1 mediated Nox2 activation inhibits glucose-induced capillary cell apoptosis in retinal endothelial cells (Kowluru et al., 2014a). We have shown that diabetic mice overexpressing *Sod2* gene have normal mitochondrial biogenesis and they are protected from the development of diabetic retinopathy (Kanwar et al., 2007). In addition, long-term administration of a stable MnSOD mimic, Tempol, ameliorates endothelial dysfunction, and MnSOD mimic, M40403, improves endoneural blood flow in the retina (Coppey et al., 2001; Nassar et al., 2002). Another MnSOD mimic, MnTBAP, protects mitochondrial homeostasis and prevents glucose-induced apoptosis of retinal capillary cells (Kowluru, 2013).

9.1.2. Human studies—Despite promising results from animal models of diabetic retinopathy, due to lack of well-designed longitudinal cohort studies and clinical trials, results from diabetic patients have been inconclusive. Calcium dobesilate (2, 5-dihydroxybenzenesulfonate, a compound with potent antioxidant capacity against hydroxyl radical), and pycnogenol (with both free radical scavenging and anti-inflammatory properties) reduces the progression of diabetic retinopathy (Garay et al., 2005; Spadea and Balestrazzi, 2001). In a small clinical trial, oral administration of α-lipoic acid with genistein and vitamins for 30 days in patients with proliferative diabetic retinopathy, has shown beneficial effects on ERG oscillatory potential (Nebbioso et al., 2012). Oral administration of vitamin E increases retinal blood flow and normalizes hemodynamic abnormalities in the retina of diabetic patients (Bursell et al., 1999). Our recent double masked, double blind, placebo controlled clinical trial, using a multi-component nutritional formula consisting of vitamins C, D3, and E, zinc oxide, eicosapentoenoic acid, docosahexaenoic acid, alpha-lipoic acid, coenzyme Q10, zeaxanthin, lutein, benfotiamine, N-acetyl cysteine, resveratrol, turmeric root extract, green tea leaf, in diabetic patients has shown clinically meaningful improvements in their visual function and in the inflammatory serum proteins. These nutrients have also reduced symptoms of diabetic peripheral neuropathy. Though these encouraging results are obtained from a limited number of patients (<70 patients), they clearly suggest the importance of regulation of oxidative stress in visual functions associated with diabetes (Chous et al., 2015). However, there appears to be no significant association between serum levels of major dietary antioxidants and

retinopathy have been reported (Millen et al., 2003, 2004). In addition, a retrospective study in type II diabetic patients, based on a single 24-h diet recall, has also shown no association between antioxidant supplementation (vitamins C and E, and β-carotene) and decrease in the severity of retinopathy (Mayer-Davis et al., 1998). Hence, although antioxidant could have potential to retard retinopathy in diabetic patients, the association between diabetic retinopathy and dietary antioxidant therapy remains elusive. There could be a number of reasons for the failure of any association between diabetic retinopathy and antioxidants as this is a progressive disease, and DCCT/EDIC studies have clearly documented that the prior damage plays a major role in the outcome of the tight glycemic control (Aiello, 2014; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group, 2005), thus the time of intervention is very crucial. A well-defined blood-retinal barrier could also impede in making the antioxidants available to the retina (Antonetti et al., 2012). However, as shown in animal models (Santos and Kowluru, 2011), the antioxidant supplements could also provide some benefit when used as an adjunct to the glycemic control for this progressive blinding disease. Thus, encouraging animal results and the limited clinical data, pave path for well-designed longitudinal cohort studies and clinical trials.

9.2. Regulation of epigenetic modifications

Current experimental data clearly suggests that epigenetic modifications have an impact on the retinopathy (Perrone et al., 2014; Tewari et al., 2012b; Zhong and Kowluru, 2011b, 2013b). Inhibition of DNA methylation by targeting Dnmt has now been tried for many chronic diseases, and the US Food and Drug Administration has already approved Dnmt inhibitors 5-azacytidine (azacitidine; Vidaza) and 5-aza-20-deoxycitidine (decitabine; Dacogen) for myeloid cancers and cutaneous T cell lymphoma. Non-nucleoside analogue RG108 is now in pre-clinical trials, and MG98 is in phase I/II clinical trials (Song et al., 2011). Furthermore, methylation of *VEGF* receptor promoter is shown to determine the efficacy of the VEGF-targeted drugs on the proliferation of cancer tissue (Kim et al., 2012). In addition to DNA methylation, therapeutic use of HDAC inhibitors has gained a lot of promise, and most of these inhibitors have strong antioxidant properties. For example, epigallocatechin-3-gallate, a strong histone acetylase inhibitor, ameliorates NF-*k*B activation (Choi et al., 2009); activation of NF-*k*B is considered to accelerate apoptosis of retinal capillary cells, suggesting this could have potential to inhibit the development of diabetic retinopathy. Resveratrol, a naturally occurring compound found in grapes, wine and eucalyptus, is a potent inhibitor of histone deacetylases (Wood et al., 2004). Natural compounds, curcumin and genistein, also modulate histone modifying enzymes, and have potential to affect the epigenetic machinery (Majid et al., 2009). As with histone acetylating/deacetylating enzymes, histone methylatransferases are also being considered as targets for therapeutics. Enhancer of H3K27 methylation enzyme is inhibited by 3-Deazaneplanocin, and specific inhibitor of EZH2, e.g., GSK126, has shown promising results for the treatment of cancer. Epigenomic-based therapies targeting histone modifications are also being developed, and they offer new approaches for the treatment of ovarian cancer (Itamochi, 2010).

As eloquently reviewed by Mastropasqua and associates, a number of miRNAs are associated with the pathophysiology of diabetic retinopathy (Mastropasqua et al., 2014). A recent report has suggested that the levels of serum miRNAs- miR-21, miR-181c and miR-1179, can discriminate between patients with proliferative and nonproliferative diabetic retinopathy (Qing et al., 2014). Double-stranded miRNA mimics and anti-mRNA antisense oligodeoxyribonucleotide are being used to target specific miRNA in other diseases, and with the recent advancements in drug delivery, the use of such modalities appears achievable.

In summary, therapies targeted towards epigenetic modifications are now being in clinical trials for other chronic diseases. Although many epigenetic modifications are implicated in the development and pathogenesis of chronic diseases, the role of epigenetic modifications in diabetic retinopathy is still evolving; this opens up the potential for therapeutics targeted towards these modifications for this sight-threatening disease.

10. Perspective and future directions

Retinopathy remains one of the most debilitating complications of diabetes with a very heavy socioeconomic burden. It affects a third of people with diabetes and the prevalence increases with the duration of diabetes with over 80% patients suffering with some form of this progressive disease after 20 years of diabetes. The pathophysiology of diabetic retinopathy is complex; a number of metabolic, physiological and functional abnormalities affect a wide range of cell types in the retina. In diabetic milieu, the microvasculatureof the retina, the major site of histopathology associated with diabetic retinopathy, experiences many metabolic abnormalities, and oxidative stress is increased. Due to consistent hit by the cytosolic ROS, mitochondria are damaged, the activity of the complex III of the electron transport chain is decreased, and mitochondrial superoxide levels are elevated. Mitochondrial DNA is damaged, and the transcription of mtDNA-encoded genes is compromised. The free radical scavenging system and mtDNA repair systems are compromised, further fueling into free radical accumulation, and the vicious cycle of free radicals continues to self-propagate (Fig. 14).

As mentioned above, epigenetics plays an important role in maintaining mitochondrial homeostasis in diabetes (Tewari et al., 2012b; Zhong and Kowluru, 2011b, 2013b). Methylation of nDNA impairs retinal mtDNA biogenesis, and that of mtDNA impairs its transcription and increases oxidative stress. Diabetes-induced histone modifications at the promoter of *MMP-9* fuel into mitochondrial damage, and that at *Sod2*, inhibit scavenging of mitochondrial superoxide radicals (Zhong and Kowluru, 2013a,b). In addition, the machinery responsible for epigenetic changes, including DNA methylation and histone modifications, is also modulated by oxidative stress; while DNA methylating enzymes and histone demethylase, LSD1 are activated in an oxidative milieu (Feng et al., 2013). Thus, oxidative stress appears to have a major role in epigenetic modifications implicated in the development of diabetes.

Human trials using antioxidants for the treatment of diabetic retinopathy, though very limited in numbers, have produced less than satisfactory results, but the promising

experimental results provide strong background for some controlled clinical trials. Also, emerging role of epigenetics in diabetic retinopathy opens up new therapeutic targets to retard/halt the progression of this devastating disease; optimistically, efforts are being put into developing the inhibitors of these modifications for the treatment of other chronic diseases.

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List of abbreviations

AGEs	Advanced glycation end-products
ALEs	Advanced lipoxidation end-products
ARE4	Antioxidant response element 4
BER	Base excision repair
Cu–Zn SOD	Copper–zinc superoxide dismutase
DCCT	Diabetes Control and Complications Trial
D-loop	Displacement loop
Dnmts	DNA methyltransferases
EDIC	Epidemiology of Diabetes Interventions and Complications
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
Keap1	Kelch-like ECH associated protein 1
LSD1	Lysine-specific histone demethylase-1
MnSOD	Manganese superoxide dismutase
mitomiRs	Mitochondrial microRNAs
Mlh1	MutL homolog 1
MMP	Matrix metalloproteinase
MMR	Mismatch repair

mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NF-kB	Nuclear factor kappa-B
Nox	Nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PARP	Poly ADP-ribose polymerase
PKC	Protein kinase C
RAGE	Cell-surface AGE-binding receptors
ROS	Reactive oxygen species
SAM	S-adenosyl methionine
Sirt1	Sirtuin 1
Sod2	Superoxide dismutase 2
Sp1	Specificity protein 1
TDG	Thymine-DNA glycosylase
TET	Ten-eleven translocation methyl cytosine dioxygenase
TEAM	Transcription Factor for mtDNA
THP-1	Tamm-Horsfall Protein 1
Tiam1	T-cell lymphoma invasion and metastasis 1
TXNIP	Thioredoxin interacting protein
UKPDS	UK Perspective Diabetes Study

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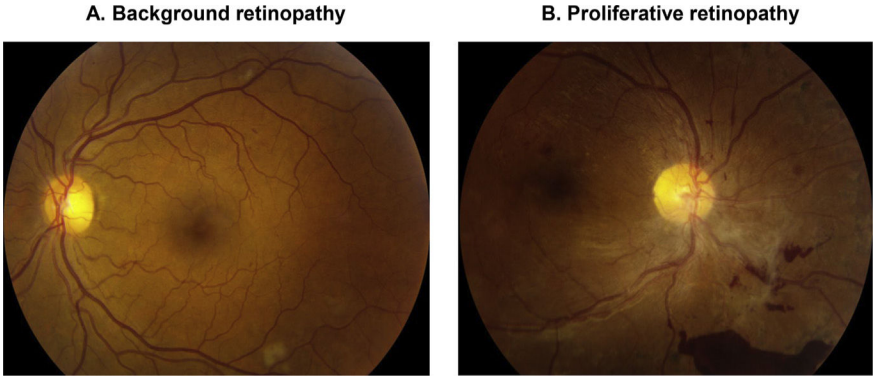

Fig. 1.

Fundus photographs of diabetic patients with A. background retinopathy showing some white patches (cotton wool spots) above the optic disc, indicating blocked small blood vessels, and a small hemorrhage. B. Proliferative retinopathy with new vessels on the optic nerve head and numerous hemorrhages.

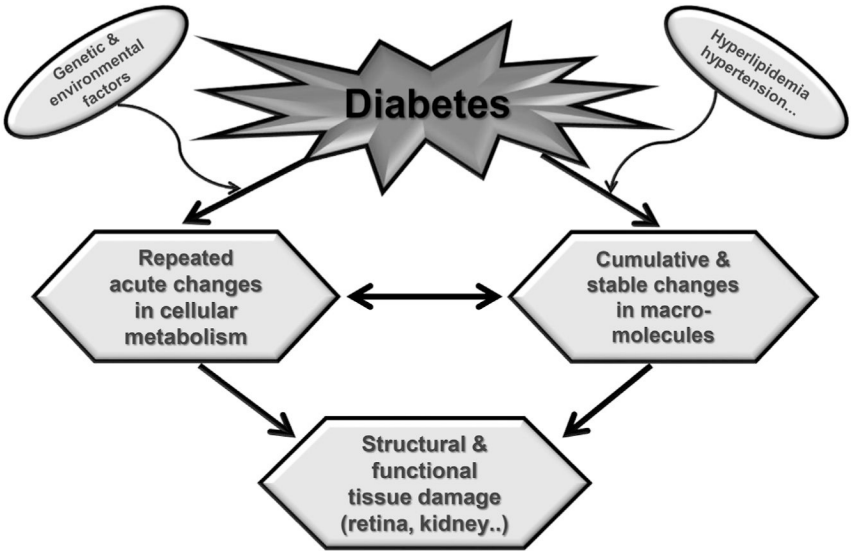

Fig. 2.

Chronic hyperglycemia can result in many acute and cumulative changes in cellular metabolism, and these can damage structure and function of many organs. Repeated acute changes in the metabolism can also produce cumulative changes in the macromolecules. In addition to hyperglycemia, genetic/environmental factors and other systemic factors (hyperlipidemia or/and hypertension) also influence the tissue damage.

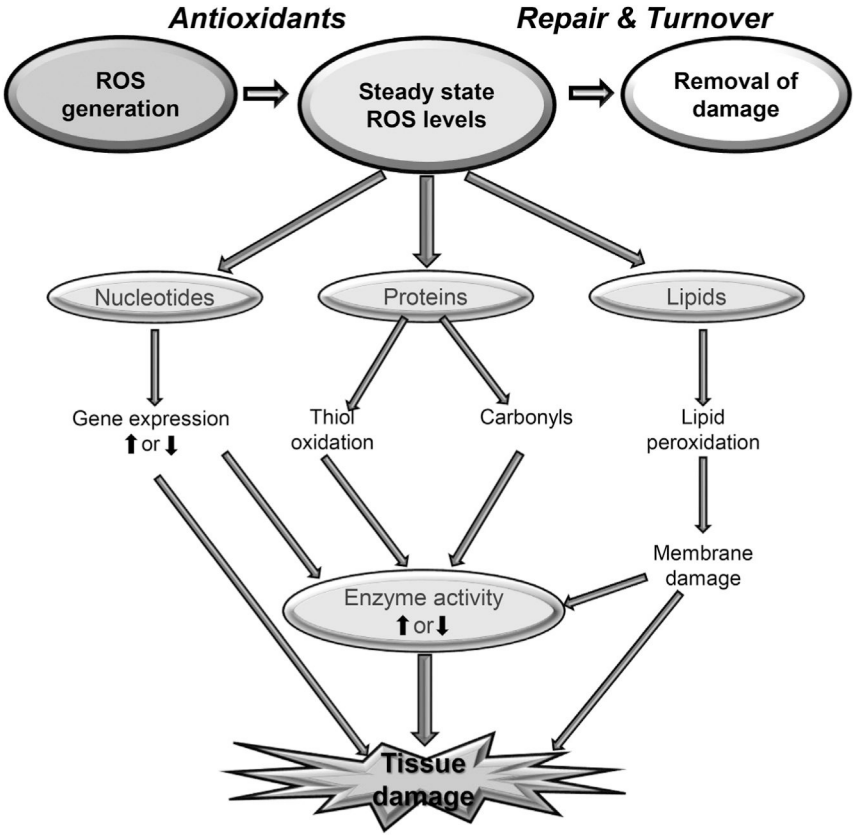

Fig. 3.

Increased ROS damage macromolecules directly via by influencing gene expressions or by damaging membrane. Damage to nucleotides, oxidation of thiols in protein, and oxidation of lipids in the membranes can also alter enzyme activity, resulting in tissue damage.

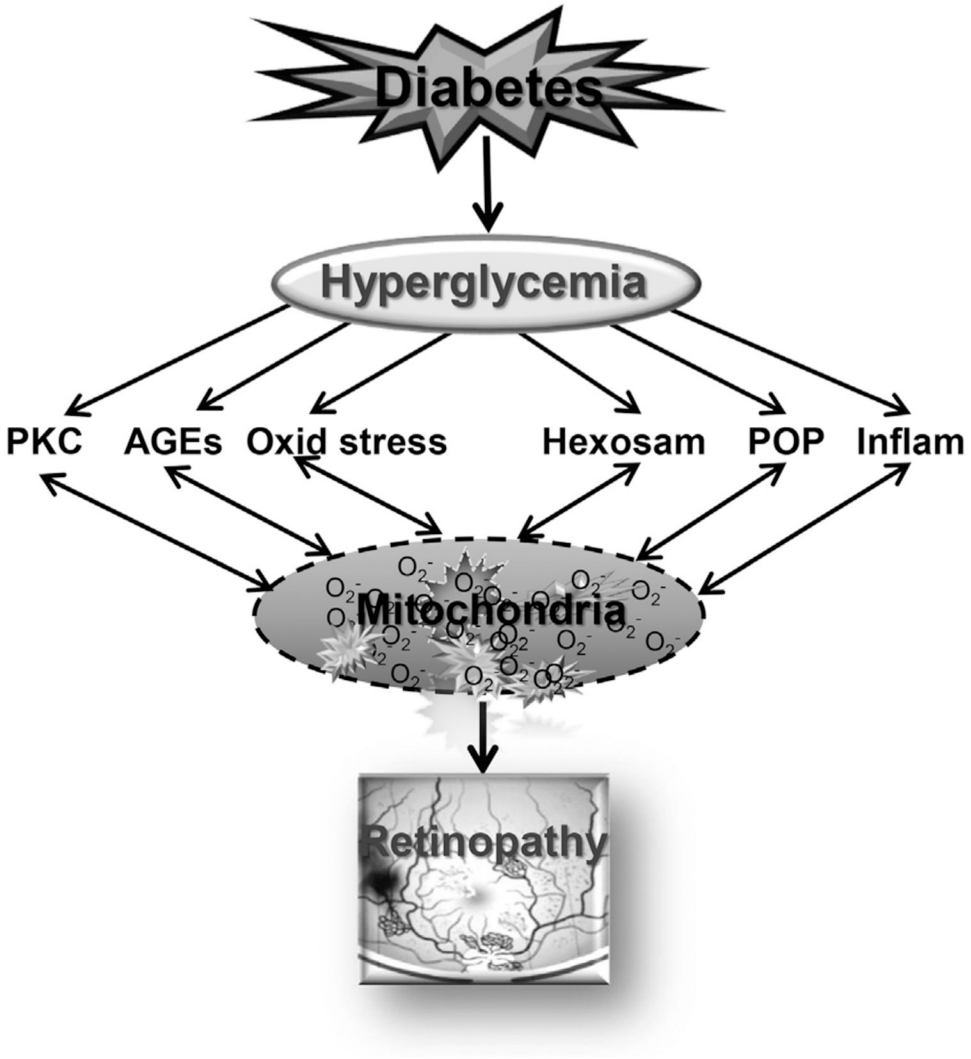

Fig. 4.

Hyperglycemic environment activates a number of metabolic pathways in the retina, including PKC, AGEs, hexosamine pathway (Hexosam), polyol pathway (POP), oxidative stress (Oxid stress) and increases inflammatory mediators (inflam). These all lead to increase in mitochondrial ROS. Increased mitochondrial ROS, instead can also activate these metabolic pathways, suggesting two way interactions between various metabolic abnormalities and mitochondrial damage. Damaged mitochondria, via accelerating apoptosis of capillary cells, result in acellular capillaries and pericyte ghosts.

Fig. 5.

The transcription factor Nrf2 controls the catalytic subunit of GSH biosynthesis enzyme glutamate cysteine ligase (GCL), and glutamylcysteine formed from glutamate and cysteine, is converted to GSH by GSH synthetase.

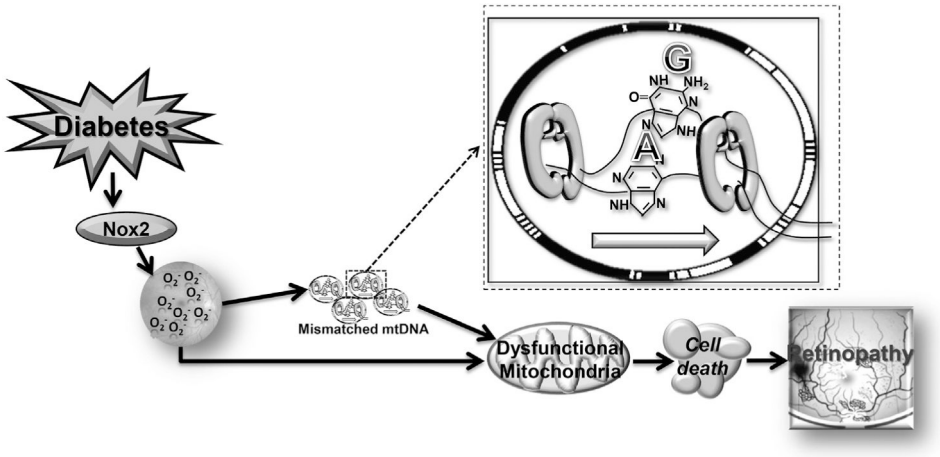

Fig. 6.

Sustained high glucose produces mismatches in retinal mtDNA, and due to suboptimal sequence repair machinery, mtDNA is damaged.

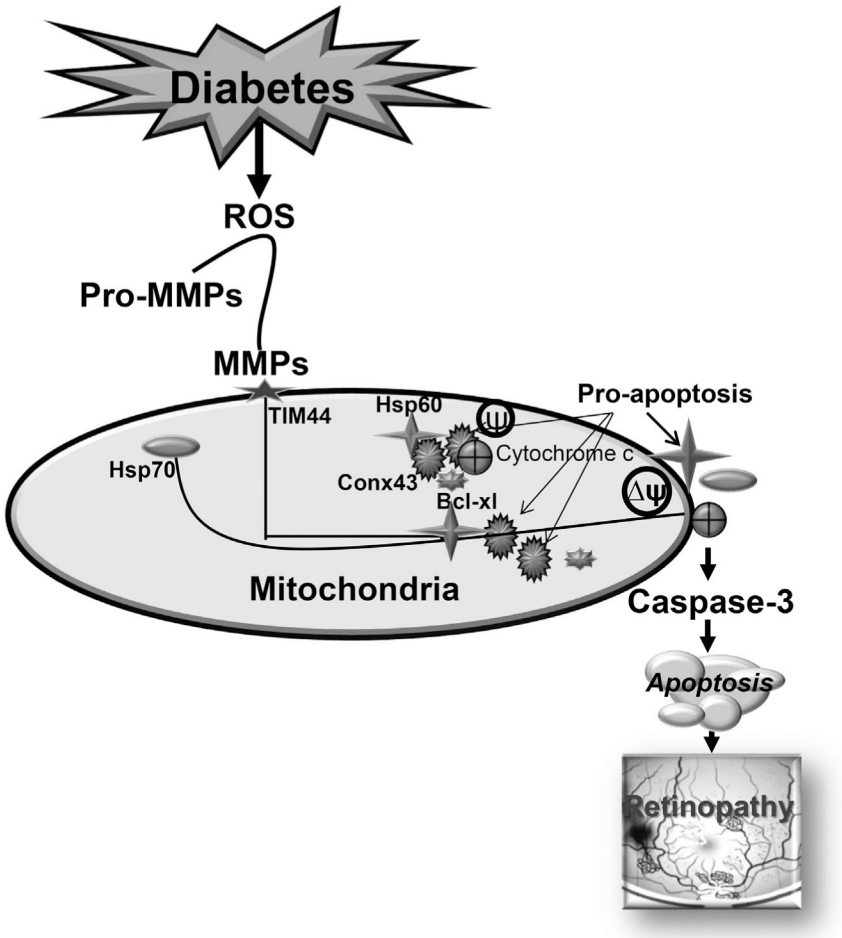

Fig. 7.

Retinal MMPs are activated by cytosolic ROS, and via mitochondrial membrane transporters (TIM44), move into the mitochondria. Inside the mitochondria, MMPs act on connexin 43 (conx43), and damage mitochondrial membrane, cytochrome c leaks out into the cytosol, and apoptotic machinery is activated.

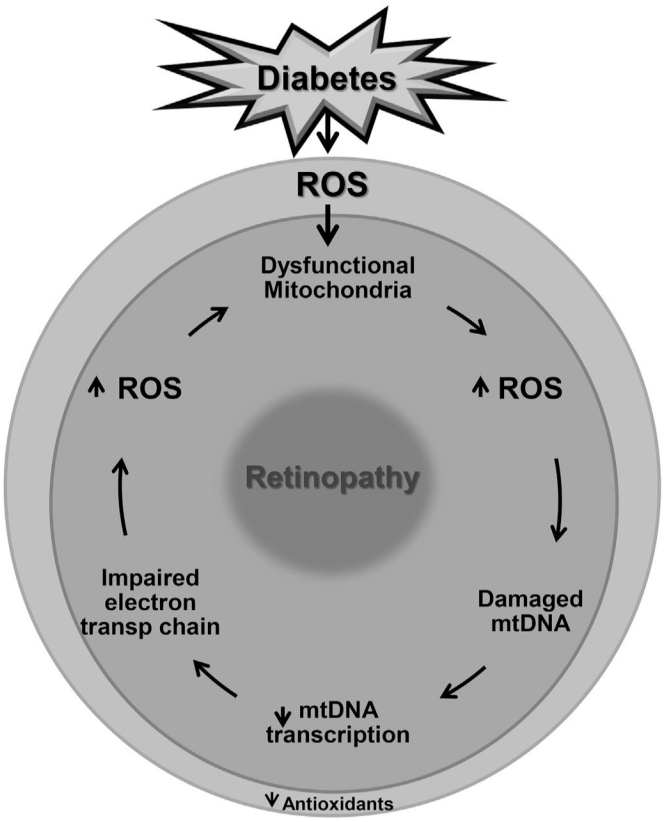

Fig. 8.

ROS produced in the cytosol damage mitochondrial function, and the levels of ROS are increased in the mitochondria. Sustained increase in mitochondrial ROS damages mtDNA, and the transcription is impaired. This leads to a compromised the electron transport chain, which further fuels into increased ROS and mitochondrial dysfunction, and the vicious cycle of ROS continues.

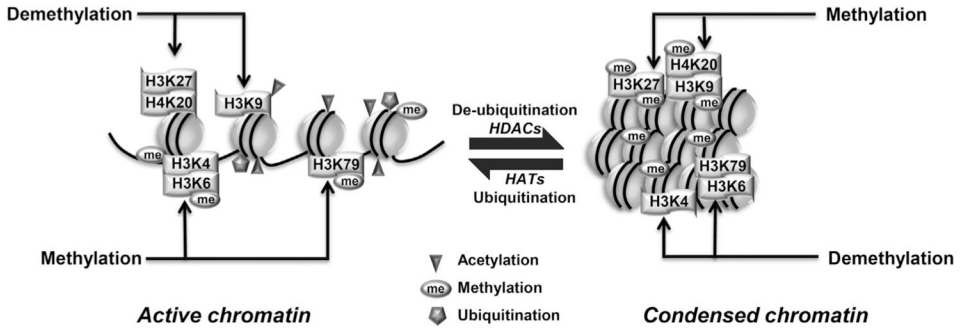

Fig. 9.

Chromatin can either be open (active, allowing gene expression) or condensed (inactive, repressing gene expression). Active chromatin is maintained by H3K9, H3K27, and H4K20 demethylation and H3K4, H3K79, H3K6 methylation, and histone acetylation or ubiquitination. Conversely, selective histone methylation (e.g., H3K9, H3K20, H3K27) results in chromatin condensation and transcriptional repression. Histone acetyltransferases (HATs) add an acetyl group, while histone deacetylases (HDACs) remove an acetyl group.

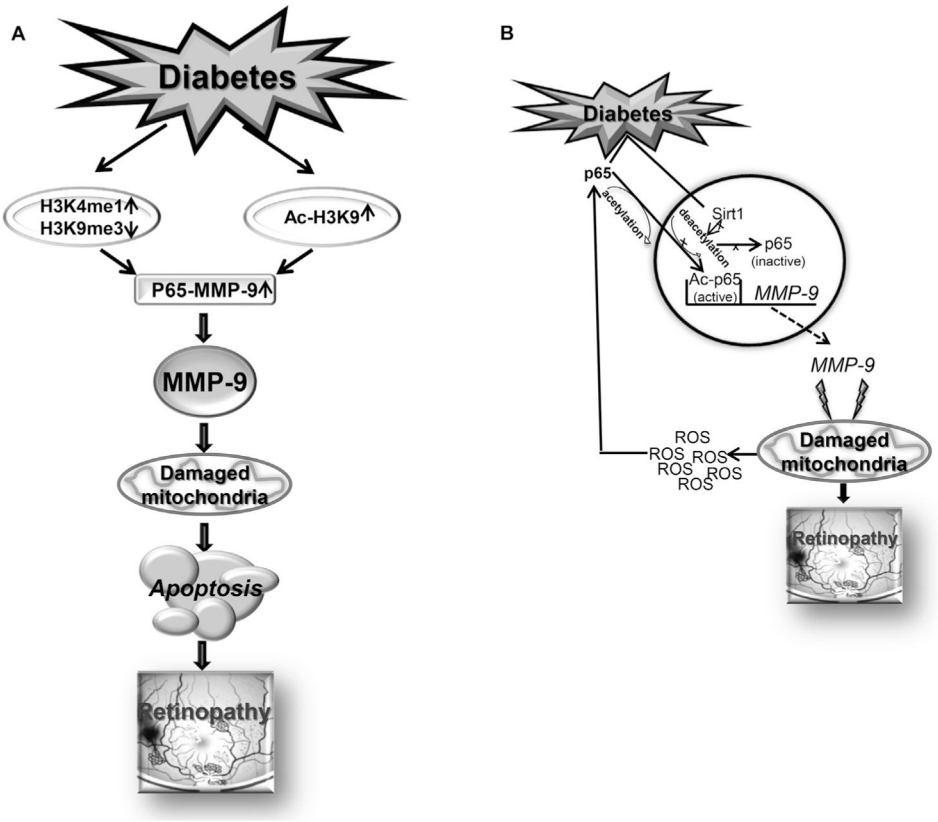

Fig. 10.

A. Diabetes increases the levels of H3K4me1 and decreases H3K9me3, and the lysine on H3K9 becomes available for acetylation. Increased Ac-H3K9 facilitates the binding of p65 at *MMP-9* promoter, and increases *MMP-9* transcription. B. Decreased levels of Sirt1 deacetylase increase acetylation of p65, and this increases it's binding at the *MMP-9* promoter. Increased MMP-9 leads to mtDNA damage and cell apoptosis in diabetic retinopathy.

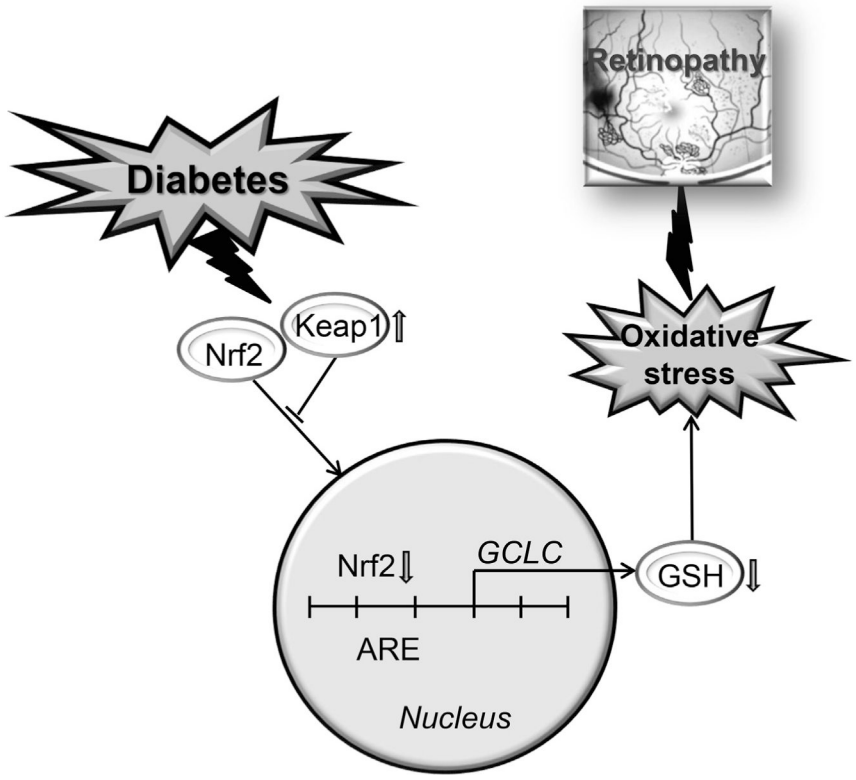

Fig. 11.

Due to epigenetic modifications at *Keap1* promoter, the binding of transcriptional factor Sp1 is increased resulting in overexpression of Keap1. Increased Keap1 restrains Nrf2 from moving into the nucleus, and the binding of Nrf2 at *Gclc-ARE4* promoter region is decreased resulting in decreased GSH biosynthesis and increased oxidative stress.

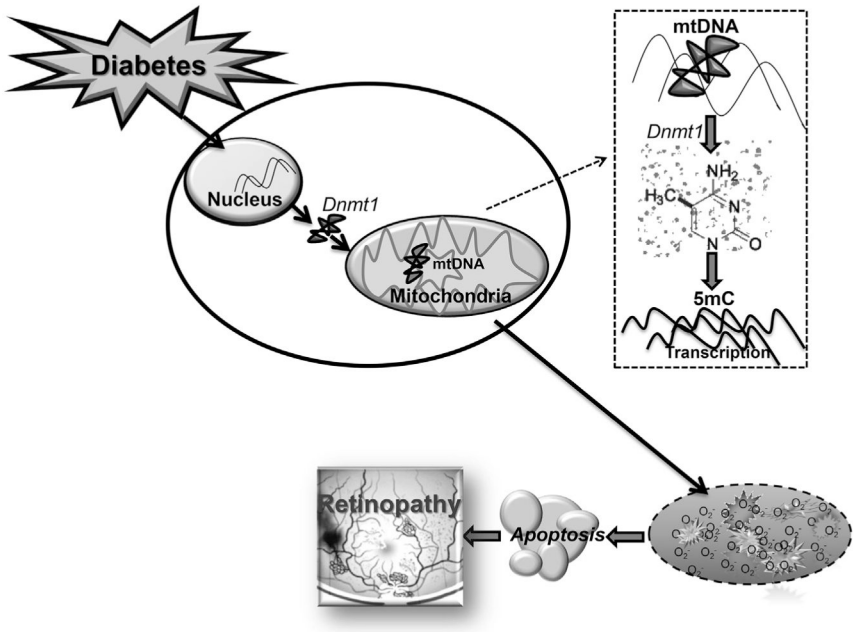

Fig. 12.

Diabetes increases translocation of Dnmt1 into the mitochondria, where it methylates mtDNA. Increased mtDNA methylation suppresses its transcription, and the electron transport system becomes compromised, further increasing ROS levels, leading to cells apoptosis.

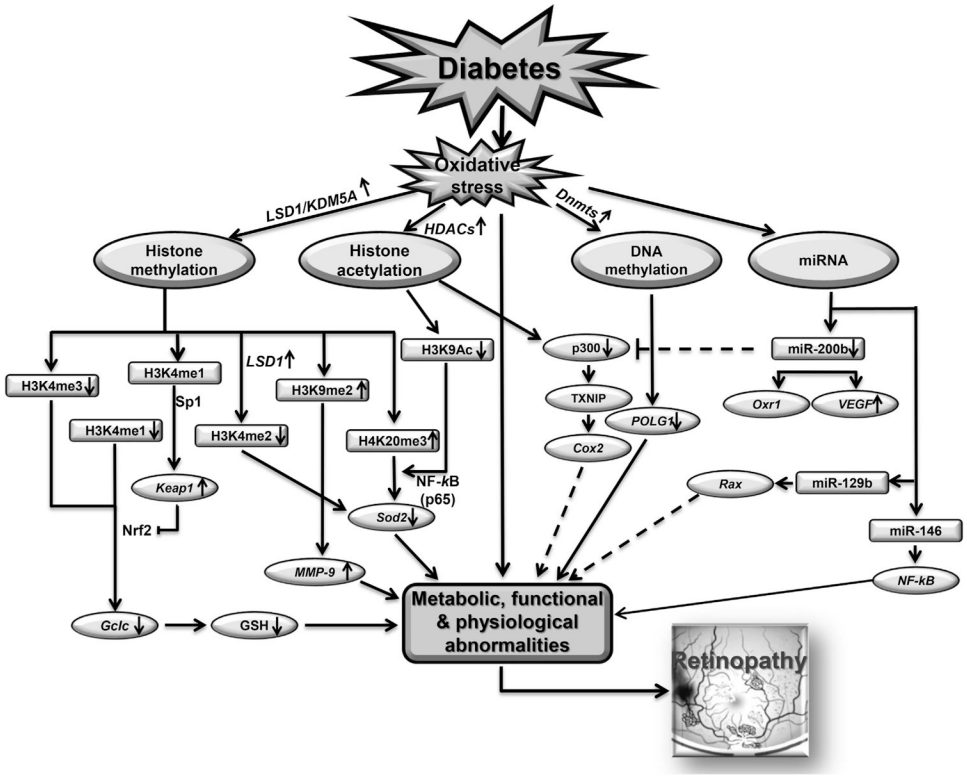

Fig. 13.

Schematic representation of epigenetic modifications in diabetic retinopathy: diabetes induces oxidative stress, which alters the expression of genes involved in histone (LSD1, KDM5A, HDACs), and DNA (Dnmts) modifications. Histone methylation (H3K4me3, HeK4me1, H3K4me2, H3K9me2, H4K20me3) and acetylation (H3K9-Ac, p300) regulates the binding of transcription factor (Nrf2, Sp1, NF-kB-p65) and alter the gene expression (*GCLC, Keap1, MMP-9, Sod2, TXNIP*), and DNA methylation at *POLG1* promoter suppresses its expression in diabetic retinopathy. MicroRNAs (miR-200b, miR-129b, miR-146) also regulate the transcript levels of various genes (*Oxr1, VEGF, Rax, NF-kB*) in diabetic retinopathy. Although this scheme represents a number of modifications, we cannot rule out the role of many, yet identified, miRNAs and other histone and DNA modifications in diabetic retinopathy.

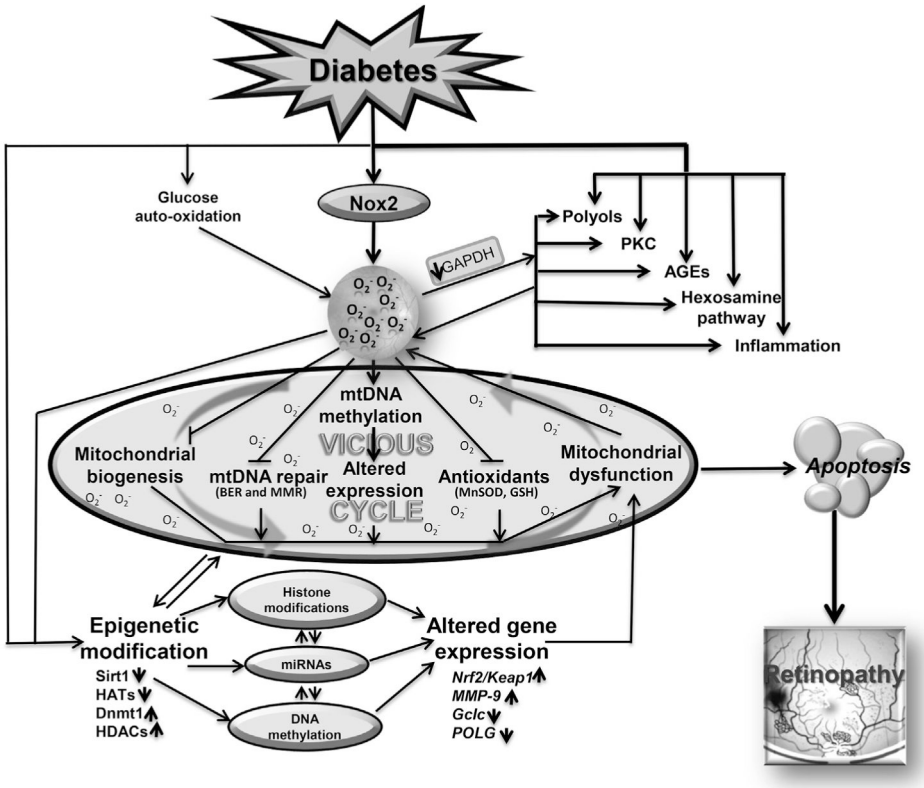

Fig. 14.

Diabetes increases oxidative stress, and this could be either via Nox2 activation or abnormal glucose metabolism, or by auto-oxidation of glucose itself. Increased oxidative stress leads to AGEs formation, activation of PKC, hexosamine, polyol pathways, and increase in inflammatory mediators. Increased ROS attenuate the GAPDH, which further activates PKC, hexosamine and polyol pathways and AGEs formation, and these pathways also can produce ROS. Epigenetic modifications in the histones or DNA alter the gene expressions of proteins associated with the oxidative damage and antioxidant defense, miRNA levels are altered, and these further dysfunction mitochondria and impair mtDNA transcription. The vicious cycle of ROS continues to fuel in, resulting in cell apoptosis and the development of diabetic retinopathy.