TARGETING SPERMINE OXIDASE TO REDUCE NEURODEGENERATION AND VISION LOSS IN DIABETIC RETINOPATHY

by

MOADDEY SALEH ALFARHAN

(Under the Direction of S. Priya Narayanan)

ABSTRACT

Diabetic retinopathy, a major cause of vision loss, is characterized by neurodegenerative and vascular changes in the retina. Current treatments target the late phase of the disease and have undesirable side effects. Previous studies from our laboratory have shown that spermine oxidase (SMOX) is critically involved in retinal neurovascular changes. However, the molecular mechanisms of SMOX-induced neuronal damage and the impact of the long-term effects of SMOX inhibition on the diabetic retina have not been investigated yet. This present study investigated the molecular mechanisms regulated by SMOX in causing retinal neuronal damage and determined the impact of long-term inhibition of SMOX on neuro-glial injury and vision loss. Utilizing experimental models of retinal excitotoxicity, our results demonstrated upregulation in the number of microglia/macrophages in excitotoxic retinas, while MDL 72527 (a pharmacological inhibitor of SMOX) treatment reduced these changes. When retinal excitotoxicity upregulated several pro-inflammatory genes, MDL72527 treatment reduced many of them and increased anti-inflammatory genes. Furthermore, SMOX inhibition upregulated antioxidant signaling and

reduced the level of conjugated acrolein in excitotoxic retinas. In vitro studies using a microglia cell line showed activated morphology and increased reactive oxygen species formation in response to the treatment with conjugated acrolein (a product of SMOX function). Streptozotocin-induced mouse model of diabetes was used to assess the effectiveness of long-term SMOX inhibition in visual acuity (VA), contrast sensitivity (CS), retinal function, and neuro-glial injury. Significant reductions in measures of VA and CS were observed in diabetic mice following 8, 16, and 24 weeks of diabetes. However, treatment with MDL 72527 improved both VA and CS at all the time points studied, with a significant increase at 24 weeks post-diabetes. Diabetic mice indicated marked reductions in the scotopic electroretinography (ERG) amplitudes, while MDL 72527 treatment significantly improved these reductions. Further, using neuronal and glial markers, MDL 72527 treatment reduced neuronal and glial injury in the diabetic retina, indicated by changes in neuronal and glial markers, respectively. Overall, our findings suggest that SMOX plays a critical role in retinal neurodegeneration and targeting SMOX signaling using MDL 72527 may provide a new strategy for reducing vision loss in diabetes.

INDEX WORDS: Spermine oxidase, MDL 72527, diabetic retinopathy, neurodegeneration, excitotoxicity, neuroinflammation, glial injury, vision

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MOADDEY SALEH ALFARHAN

B.Pharm, King Khalid University (Saudi Arabia), 2012

MS, St. John's University, 2018

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MOADDEY SALEH ALFARHAN

Major Professor:	S. Priya Narayanan
Committee:	Mohamed Al-Shabrawey
	Somanath Shenoy
	Duo Zhang

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. Diabetic retinopathy

According to the Centers for Disease Control and Prevention (CDC), vision loss ranks as one of the top ten disabilities in adults in the United States (U.S.) [2]. In the U.S., more than 12 million Americans aged 40 years and older have vision impairment, including one million who are legally blind [3]. Vision loss might prevent patients from practicing daily activities such as reading, driving, or watching television. The primary diseases in the U.S. that lead to blindness and low vision are age macular degeneration, cataracts, diabetic retinopathy, and glaucoma [3]. According to CDC, 11.3% of the U.S. population has diabetes, and 38% of the U.S. population over 18 years are prediabetic [4]. However, almost 50% of diabetic patients are not getting an eye exam, making them late for effective treatment [3]. Diabetic retinopathy (DR) is a significant public health issue and the leading cause of vision loss among working-age adults [5]. DR is a common complication of diabetes, present in almost all patients with diabetes for more than 15 years [6]. Among American adults with diabetes, 28.5% have DR [7]. In 2020, the number of adult patients with DR worldwide was more than 103 million, which is expected to reach more than 160 million by 2045 [8,9].

DR progression is affected by various factors, including the duration of diabetes, age, pregnancy, hyperglycemia, hyperlipidemia, obesity, smoking, etc. [10]. The clinical diagnosis of DR usually depends on changes in the retinal vasculature. The vascular changes being used to define the disease stage include microaneurysms, intraretinal hemorrhage, and neovascularization [11]. Although DR is clinically considered a microvasculature defect, increasing evidence supports that neurodegeneration plays a critical role, and it might occur before the micro capillaries are affected [12-19]. DR is now recognized as a neurovascular disease, and retinal neurons play a major role in the progression of DR [20-23]. Studies from diabetic animal models have also shown that in addition to the vasculature, retinal neurons are also affected by diabetes, causing alterations in visual functions [24]. Some of the common signs of retinal neuronal dysfunction in DR patients include loss of color vision and contrast sensitivity within two years of diabetes [25-27].

The vision loss associated with DR affects patients' quality of life and has negative social and psychological effects. Current treatments for DR are mainly for advanced stages where PDR or diabetic macular edema (DME) has already developed, and neuronal damage has already occurred [28]. Therefore, it is necessary to find a better intervention to protect against vision loss or slow disease progression for diabetic patients.

2. Structure of retina and organization of retinal layers

The retina is the innermost layer in the eye responsible for proper vision. It consists of multiple cell types (neurons, glia, and endothelial cells), divided into ten layers, each playing a specific role in transmitting vision [29]. There are five types of neurons in the retina. These include photoreceptors, bipolar, horizontal, amacrine, and ganglion cells [30]. Retinal neurons in different

layers are interconnected by synapses (Figure 1.1) [31]. About 95% of human photoreceptors are rods that help to create black-and-white vision [29]. The other 5% of photoreceptors are cones that detect light colors [29]. Bipolar cells, which receive visual inputs from photoreceptors (rods and cones) and project their axons onto retinal ganglion cells, have thirteen different types depending on which input they receive [29]. Horizontal cells have three distinct types. Their main function is modulating the transfer of information between bipolar cells and photoreceptors and helping the eye adjust to both bright and low light conditions [29]. Amacrine cells are intermediate neurons responsible for inhibitory neurotransmitters (GABA and glycine) [32]. Finally, retinal ganglion

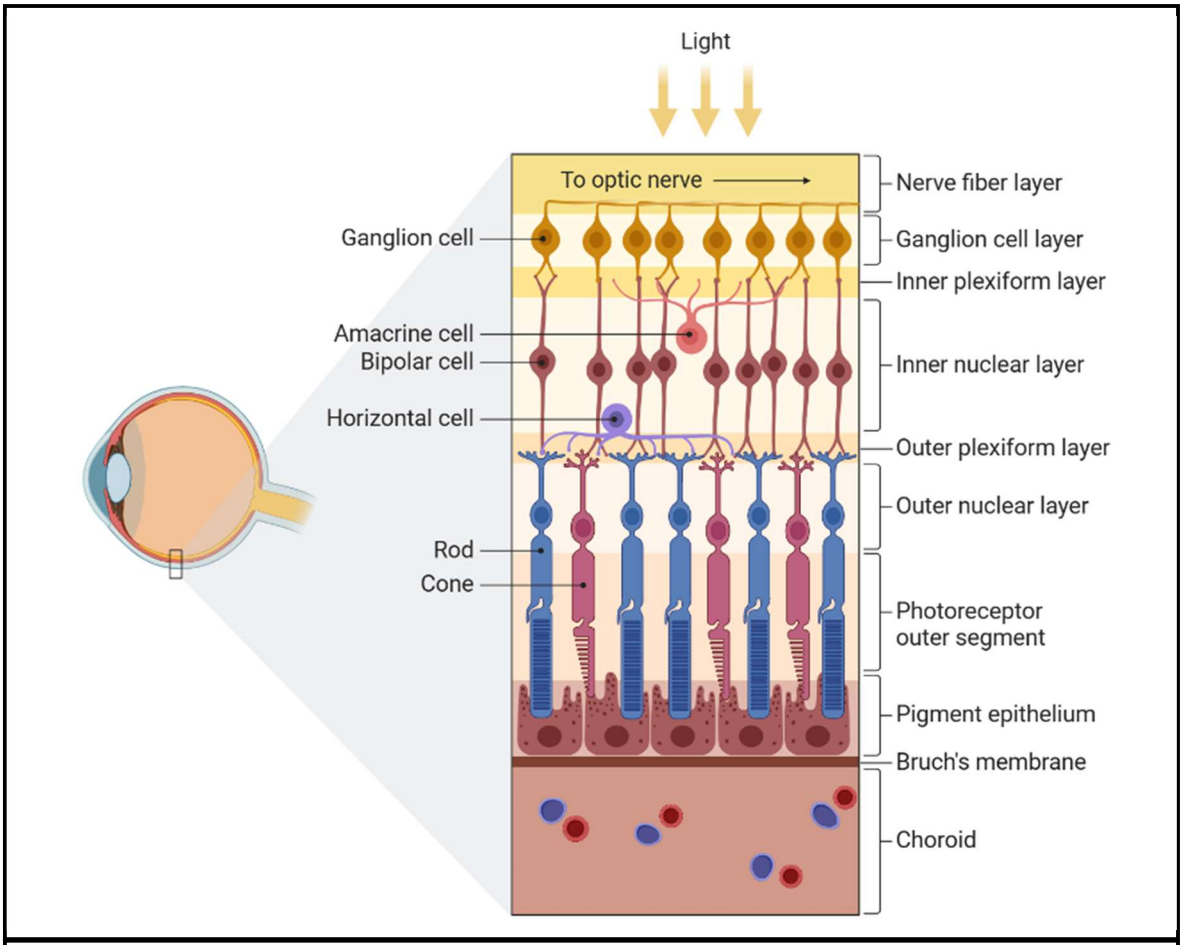


Figure 1.1. The structure of the retina. This figure shows retinal layers and retinal neuronal cell type. Created in BioRender.com

cells, which are the retina's main output neuron, receive excitatory and inhibitory inputs from the two intermediate neurons, amacrine cells, and bipolar cells [29].

3. Neurodegeneration in DR

Neurodegeneration is a significant hallmark of DR progression in both types of diabetes [33,34]. Retinal neuronal cells are fragile and highly demanding elements, and they get affected early when the environment is changed [35]. It is reported that neuronal apoptosis in the diabetic retina occurs at the early stage of the disease, and the duration of diabetes increases the rate of neuronal apoptosis in experimental diabetic retinopathy [36]. Furthermore, the proapoptotic molecules are observed to be increased while the survival molecules decrease in the retina of diabetic patients with mild or no vasculature changes [37].

3.1. Signs of neurodegeneration in DR

Several signs of neuronal damage and dysfunction are observed during the progression of DR in patients and experimental models, and these are discussed in the sub-sections below.

3.1.1. Loss of visual acuity and contrast sensitivity

Reduced color vision, contrast sensitivity, and visual acuity in diabetic patients are reported as early signs of dysfunction of retinal neurons in diabetic patients that occur within two years of diabetes [25-27]. Visual acuity and contrast sensitivity tests are proper measurements to evaluate visual impairment in the eye of diabetic patients [38]. Contrast sensitivity is important for edge discrimination [39]. The reduction in contrast sensitivity reduces the quality of life and increases the risk of accidents [39]. In diabetic mice, reductions in visual acuity and contrast sensitivity are

shown to start as early as after two months of diabetes and persist for at least ten months of diabetes [40-42]. In STZ-diabetic rats, the reduction in visual acuity was significant after three weeks of diabetes, but the reduction in contrast sensitivity was significant after nine weeks of diabetes [43,44]. Furthermore, that decline in visual acuity and contrast sensitivity increased with hyperglycemia duration [43,44].

3.1.2. Alterations in retinal function

Damage to the neuroretina produces functional abnormalities, and these alterations can be detected by electroretinography (ERG) analysis. ERG is a standard method to study retinal neuronal function in cases of diseases or trauma [45]. ERG records the retinal response to brief flashes. The a-wave is a negative wave originating at the level of the photoreceptors (rods and cones). This negative wave is followed by a b-wave which is a positive wave that originates in the mid-retina [45]. Studies have shown that retinal function is severely affected in DR patients and experimental models [46,47]. Studies from our laboratory showed that streptozotocin (STZ)-induced diabetic mice showed a reduction in the retinal neuronal function starting from week four after diabetes [48]. Another study using an experimental model of type 2 diabetes (db/db) showed slight changes in a-waves and b-waves in eight-week-old diabetic mice, with noticeable in 16 and 24 weeks [49].

3.1.3. Retinal thinning

Another sign of retinal neurodegeneration that has been noticed at the early stages of the disease is the reduction in retinal thickness. Reports from patients with type 1 diabetes (T1D) or type 2 diabetes (T2D) have shown that the thickness of the total retina, retinal nerve fibers layer (RNFL), ganglion cell layer (GCL), and inner nuclear layer (INL) were reduced [50-56]. The reduction in

the total retinal thickness, ganglion cells complex (GCC) including (RNFL+GCL+IPL), and outer nuclear layer (ONL) was also noticeable in the STZ-diabetic model and db/db mice, suggesting neuronal cell loss in these layers [48,49,57,58].

3.1.4. Loss of retinal ganglion cells

Retinal ganglion cells (RGCs) are damaged during DR progression, leading to vision loss [59]. RGCs are essential because they are responsible for the entire retinal signals' output in their long axons to the brain in vertebrates [60]. Retinal samples from the human postmortem samples showed increased TUNEL-positive RGCs [36]. Also, using TUNEL staining, STZ-diabetic rat sections showed a significant increase in the number of apoptotic cells, mainly in the RGC layer, after one month of diabetes [36]. STZ-diabetic rats also showed a significant increase in apoptotic ganglion cells after 15 weeks of diabetes [61], indicating the presence of chronic neurodegeneration. Apoptotic markers, including caspase-3, caspase-9, Fas, and Bax, were also increased in RGCs of diabetic retinas [58,62-64].

While many ocular diseases affect RGCs, diabetes is an exacerbating risk factor [59]. RGC death in the diabetic retina occurs in response to hypoxia [65], inflammation [66], or oxidative stress [67]. Some other factors contributing to RGC death include increased levels of excitotoxicity molecules such as homocysteine [68] and glutamate [69]. Excitotoxicity activates the N-methyl-D-aspartate receptor (NMDA), abundantly expressed in RGCs [70]. The neuronal overstimulation through NMDA receptors leads to the death of RGCs [69].

3.1.5. Degeneration of other retinal neurons

In addition to RGCs loss, diabetic mice demonstrate a significant reduction in the number of amacrine cells affecting retinal function [71,72]. The retinas of diabetic rats exhibited degeneration of the photoreceptor layer [73]. Using an electroretinogram (ERG), a clinical study has shown that rod and cone a-wave and b-wave sensitivity were changed in the retina of diabetic patients with a history of diabetes for five years or more [74]. Another clinical study showed that patients newly diagnosed with type 2 diabetes without any vascular changes exhibited an alteration in the function of both rods and cones [75].

Photoreceptors consume more oxygen than other cells, especially in the dark [76]. Therefore, they become more susceptible to hypoxia when vasculature of the retina is compromised, such as in diabetes [77]. Photoreceptors are major retinal cell type enriched in mitochondria, which might cause increased reactive oxygen species production [77,78]. They are the major sites in retina generating superoxide in diabetes, which worsens during the dark [79]. Oxidative stress caused by photoreceptors induces the production of pro-inflammatory cytokines, which develop microvascular changes during diabetes [79]. In addition to the retina, the optic nerve is also affected, presenting myelin loss, axonal degeneration, and optic nerve gliosis [71,80].

3.2. Mechanisms of diabetes-induced neuronal damage in the retina

Several mechanisms/signaling pathways have been demonstrated to be involved in the pathogenesis of DR [81,82]. These mechanisms contributing to retinal neuronal damage include oxidative stress, inflammation, and reduction in neuroprotective factors.

3.2.1 Oxidative stress

Studies have documented that oxidative stress plays a critical role in the pathogenesis of DR [83]. Hyperglycemia can activate different metabolic pathways such as polyol, hexosamine, protein kinase c (PKC), and advanced glycation end products (AGEs) [10]. The accumulation of byproducts of these pathways can induce oxidative stress by forming reactive oxygen species (ROS) and nitrogen-oxygen species (RNS) and decreasing the level of antioxidants [10]. The increase in ROS and RNS and the reduction in antioxidants can cause severe damage to neurons in retinal tissue by activating several metabolic and apoptotic pathways [84]. ROS formation has been shown to play an essential role in modulating cell survival, cell death, differentiation, cell signaling, and inflammation [85]. Clinical studies showed that an increase in ROS production might be responsible for the rise in glutamate levels to the neurotoxic level in the vitreous [86]. The increase in ROS production and hypoxia exposure in the diabetic retina contribute to angiogenesis and neurodegeneration in DR [87]. One clinical study that supports that oxidative stress in DR is crucial in disease pathogenesis has shown that a high dietary intake of antioxidants, including vitamin E and selenium, appeared to have a protective effect on DR [88]. Even though the role of oxidative stress in diabetic retinopathy has been well established, the specific mechanisms of oxidative stress-induced neuronal abnormalities in the diabetic retina are not completely understood.

3.2.2. Excitotoxicity

Glutamate neurotoxicity is known as a major mechanism of neurodegeneration in the CNS [89]. Studies showed that diabetes alters Müller cells' function, which is responsible for glutamate metabolism. The reduction in glutamate metabolism leads to increased glutamate levels in the retina after short-term diabetes [90]. Glutamate is the major excitatory neurotransmitter in the

retina. However, the increase in glutamate levels is considered neurotoxic and causes neuronal death in many neurodegenerative diseases [91]. Increased level of glutamate is found in the retina of experimental animal models [90,92,93] and vitreous fluid of diabetic patients [94,95] and correlated with the inability of macroglia to metabolize the glutamate [90]. Chronic low-dose glutamate injections to mimic the increase of glutamate in the retina resulted in the loss of 42% of retinal ganglion cells after three months [96]. There are two main types of glutamate receptors inotropic and metabotropic. Ionotropic glutamate receptors, which are responsible for the excitatory transmission in the CNS, are divided into three main classes: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, 2-carboxy-3-carboxymethyl-4-isopropenyl-pyrrolidine (kainite) receptors, and NMDA receptors [97]. NMDA receptors are the ligands of glutamate and the primary excitatory neurotransmitter in the retina [98]. Neurons in the GCL, including ganglion and amacrine cells, are most affected because of the overstimulation of the NMDA subtype of glutamate receptor [69,96]. Homocysteine is a homolog of cysteine produced by methionine's demethylation that usually increases in patients with DR [99]. Homocysteine also activates NMDA receptors and induces neuronal death in-vitro [100-102]. An in-vivo report showed that the increase of homocysteine in plasma was associated with the acceleration of retinal neuronal death in diabetic mice [68]. NMDA receptors mediate toxicity in retinal ganglion cells by increasing the influx of extracellular Ca^{+2}. Increasing intracellular Ca^{+2} activates protein kinase C (PKC), phospholipases, proteases, endonucleases, nitric oxide synthases, and free radical production [69]. The increase of intracellular Ca^{+2} also causes mitochondrial failure and releases cytochrome C that mediates the apoptosis process through caspase-3 and caspase-9 [103]. The influx of Na^{+} and Ca^{+2} ions is associated with an influx of water, leading to neuronal swelling and lysis in a process called necrosis [103]. Also, the activation of glutamate receptors can induce

proapoptotic protein p53 in the neuronal cells by activating proapoptotic Bax signaling and suppressing cytoprotective Bcl-2 signaling [104]. The inhibition of glutamate activity has protective effects on the diabetic retina by reducing apoptotic signals and ROS formation [84,96,105].

3.2.3. Neuroinflammation

In addition to neuronal and vascular changes, the diabetic retina stimulates immune cells and increases pro-inflammatory cytokines [100-102] and glial activation [106,107]. Normally, inflammation is considered a beneficial response against pathogens or tissue damage [108], but prolonged exposure to inflammation in diseases such as diabetes is believed to be involved in the pathogenesis of diabetic retinopathy [109]. Clinical studies have shown that inflammation plays a crucial role in the pathogenesis of DR by increasing pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in the vitreous of patients with DR [110-112]. One clinical study has shown that IL-6 level was raised in the vitreous of diabetic patients, and that increase was correlated with the severity of the disease [113]. Furthermore, clinical studies showed that patients with arthritis have less proliferative diabetic retinopathy (PDR) than non-arthritis, which could be because of the effects of the anti-inflammatory agents used for arthritis or the arthritis pathophysiology itself [114,115].

Glial cells in the central nervous system (CNS) play a critical role in neuroinflammation. Their primary function is to provide neuronal support. When CNS homeostasis is disrupted by neurodegeneration, inflammation, or excitotoxicity, glial cells become activated and subjected to reactive gliosis [108]. Glial cells in the retina include two types. These are macroglia (including

Müller cells and astrocytes) and microglia. These two types differ in morphology, distribution, and pathophysiology [116].

3.2.3.1. Changes in macroglia (Müller cells and astrocytes)

Müller cells, which span through the whole width of the retina and make contact with both blood vessels and neurons, are essential in the retina [117]. The Müller cell is the principal glial cell in the retina which is necessary to maintain homeostasis in the extracellular environment. Müller cells have three basic functions in the healthy retina. First, Müller cells uptake and recycle neurotransmitters, retinoic acid compounds, and ions [117]. The regulation of the glutamate/glutamine cycle by Müller cells protects retinal neurons from excitotoxicity [117,118]. Secondly, they control metabolism and supply nutrients in the retina, such as glycogen and ATP production [117]. Third, regulating blood flow and maintaining the blood-retinal barrier is another important function of Müller cells. [117].

Müller cell dysfunction plays a critical role in the pathogenesis of DR [119]. It is known that Müller cells become activated in the retina during diabetes, and the expression of glial fibrillary acidic protein (GFAP) is increased in the Müller cells and is a characteristic feature of the diabetic retina [117]. Müller glial cells, like other glial cells, undergo reactive gliosis in response to acute or chronic injury [108]. Müller cell gliosis is characterized by proliferation, changes in cell shape, and secretion of signaling molecules such as vascular endothelial growth factor (VEGF) [108,120,121]. Müller cells can uptake and degrade glutamate through glutamate transporters called glutamate aspartate transporter (GLAST), which can clear extracellular glutamate and convert it to inactive metabolites [69,117]. However, glutamate transporters GLAST were significantly reduced in the retina of diabetic animals [122]. In addition to the reduction of GLAST, glutamine synthetase that

converts glutamate to less toxic metabolites glutamine also decreased in the retina of diabetic patients [123].

However, glutamate toxicity in diabetic retinopathy is not only because of the reduction in glutamate uptake but also because of the reduction in K^+ uptake in Müller cells [117]. Changes in potassium channels in Müller cells during diabetes cause the accumulation of AGEs [124]. Disruption of potassium channels in Müller cells also decreases fluid removal and causes fluid accumulation, leading to macular edema [117].

In addition, Müller cells under hyperglycemia and hypoxia conditions increase VEGF release, which has two different effects. In one scenario, VEGF release under hypoxic conditions has a neuroprotective mechanism [125]. The other scenario is that sustained release of VEGF may act as a pro-inflammatory and causes an increase in the release of inflammatory cytokines, which ultimately aggravates retinal neurodegeneration [126]. VEGF expression stimulates angiogenesis and reduces the release of pigment epithelium-derived factor (PEDF), an angiogenesis inhibitor [127-129]. This increase in VEGF/PEDF ratio is contributed to the pathogenesis of DR [127-129]. Müller cells also produce pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in response to hyperglycemia [130-132].

Diabetic mice showed a reduction in the number of astrocytes and the decreased expression of astrocytes gap junction in the retina [133,134]. This reduction in the number of astrocytes affected the retinal function. However, diabetic rats showed a significant increase in the gliosis and proliferation of glial cells in the optic nerve [80]. Furthermore, immunostaining experiments revealed that the proliferation of astrocytes is associated with the loss of myelin in the optic tissues of diabetic rats [80].

3.2.3.2. Changes in microglia

Retinal microglia play a role in the immune defense system by releasing neuroprotective and anti-inflammatory factors. A clinical study showed that ramified microglia cells were present in the inner retinal layers; however, in the diabetic retina, microglia cells were noticeably increased and showed hypertrophic and amoeboid morphology at different stages of the disease [135]. In pre-proliferative DR, hypertrophic microglia clustered around the cotton-wool spots and dilated venules in the ganglion cell layer [135]. The number of infiltrated amoeboid microglia was increased in optic nerve heads [135]. In the PDR stage, the number of reactive microglia markedly increased in the optic nerve head region, GCL, and around the new vessels in the nerve fiber layer [135]. Another study also found a significant increase in the mRNA levels of ionized calcium-binding adaptor molecule-1 (Iba-1), the cluster of differentiation-74 (CD74), and inducible nitric oxide synthase (iNOS) in the retinas of diabetic donors [136].

Chronic hyperglycemia results in the generation of advanced glycation or lipoxidation end products (AGEs or ALEs), which causes the presence of high levels of pro-inflammatory cytokines to induce microglia activation [137-139]. In an experimental model using db/db mice, mRNA levels of iNOS, an M1 marker, and arginase, an M2 marker, were elevated during the early stage (5 weeks) of DR [136]. These markers were localized mainly in the three-layer IPL, OPL, and GCL, where microglia were immunodetected [136]. In the same study, the retinas of diabetic mice at eight weeks showed a reduction of arginase 1, but the expression of iNOS, IL6, and TNF-α mRNA levels was still high [136]. These results reveal that diabetic mice lose the anti-inflammatory defense in the retina and worsen their visual function depending on the increase with age. At the same time, there was an increase in caspase-1 in week 8 of diabetic mice compared to non-diabetic mice, suggesting the inflammasome system's involvement during DR [136]. In addition to the role of microglia in inflammation, microglia also are considered a major source of ROS formation in

CNS [140]. The increase in ROS production and the hypoxia exposure in the diabetic retina contributed to angiogenesis and neurodegeneration in DR [87].

Microglia are generally activated when damage-associated molecular patterns (DAMPs), such as S100 proteins, ATP, high mobility group box protein (HMGB1), mROS, etc., are released from the injured cells as neurons. These DAMPs interact with pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGE) on microglia [141,142]. During diabetes, the level of AGE, ALE, and oxidized LDL was increased, which causes microglial activation in the retina in different stages [143]. In the diabetic retina, nuclear factor-kappa B (NF-κB) is critical for developing inflammation in DR and other diseases [144,145]. The NF-κB pathway in DR is activated by different pathways, such as the interaction between AGE and RAGE [146] or the activation of TLRs (TLR2 and TLR4) by HMGB1 [59,147,148]. Also, the NF-κB pathway is activated in the diabetic retina through Notch signaling [149]. The activation of NF-κB signaling in the diabetic retina can modulate the expression of several pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecules-1 (ICAM-1), in addition to chemokines (C-C motif) ligand 2 (CCL2), CCL5, and CCL12 [150-153].

Microglia not only interact with neuronal cells but also with Müller cells. Wang and his colleagues have studied the crosstalk between microglia and Müller cells [154]. Their study showed that activated microglia altered the morphology of Müller cells and increased the mRNA expression of inflammatory factors IL-1β, IL-6, and iNOS [154]. This increase is likely a response to cytokines released from activated microglia, including IL-1β, IFN, and TNF-α [155-158]. On the other hand, microglia treated with the conditioned medium of Müller cells expressed higher IL-1β, IL-6, and iNOS, indicating that Müller cells have the ability to amplify the inflammatory

response in the retina [154]. Activated microglia also induced expression of VCAM-1 and ICAM-1 in addition to chemokines, including chemokine CCL2 and CCL3 of Müller cells, which increased microglial cell migration [154]. Additionally, activated microglia decreased the expression of essential factors of Müller cells that protect the retina, including transforming growth factor-beta (TGF-β), which is a necessary factor against the progression of DR [159], and GLAST, which is responsible for glutamate uptake in Müller cells [154].

3.2.3. Neuroprotective factors

Loss of neuroprotective factors or the reduction in their effectiveness in the retina is another cause of neurodegeneration. Both microglia and macroglia regulate the survival of neurons through neurotrophic factors, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and basic fibroblast growth factor bFGF [160]. Several studies have shown that reducing the activity of growth factors and neurotrophic factors such as NGF, BDNF, insulin, and platelet-derived growth factor subunit-b (PDGF-b) reduces survival signaling and increases the apoptotic signaling in the diabetic retina [61,161-164].

3.3. Current treatments

The existing treatments for DR aim at reducing the microvascular complications. These mainly include laser photocoagulation, anti-VEGF therapy and use of corticosteroids. Data from the Early Treatment Diabetic Retinopathy Study (ETDRS) showed that pan-retinal photocoagulation (PRP) reduced the risk of severe vision loss by 50% in patients with high risk of PDR [165]. On the other hand, PRP increased the risk of worsening macular edema in patients with pre-existing macular edema at the onset of treatment [166]. In addition to deteriorating macular edema, PRP treatment might cause visual field loss, loss of color vision, reduction in night vision, reduction in contrast sensitivity, and causes choroidal complications [167].

Anti-VEGF agents, including aflibercept, bevacizumab, and ranibizumab, effectively improved visual acuity in patients with DME after 1 and 2 years of treatment [168,169]. Although the beneficial effect of anti-VEGF agents, some patients showed moderate or poor response to the treatment [170]. In experimental models of DR, inhibition of VEGF increased neuronal apoptosis and reduced the number of RGCs and amacrine cells in addition to worsening the visual acuity [171,172]. In contrast, VEGF treatment increases neuronal survival the inhibition of VEGF in diabetic mice [171]. Dexamethasone and fluocinolone acetonide (FAc) effectively treat DME [173,174]. However, intravitreal injections of steroids have side effects such as cataracts and glaucoma [175].

Overall, all these treatments are expensive, require retinal specialists, and have undesirable secondary side effects. Hence there is a great need for novel pharmacological targets for the early stages of DR.

3.4. Potential therapeutic approaches targeting neurodegeneration in DR

Until now, there has been no approved treatment for targeting neurodegeneration in the diabetic retina. However, several interventions with possible neuroprotective effects are being investigated. Following are some of these interventions that target retinal neurons.

3.4.1. Anti-hyperglycemic agents

Insulin has prosurvival actions in the retina and can protect retinal neurons during diabetes [84]. Neuronal cell death in the diabetic retina is associated with reducing insulin receptor kinase activity [57]. The increase of apoptotic neuronal cells in the retina in insulin-deficient diabetic rats was reversed by systemic insulin treatment [36]. Insulin treatment in diabetic animals also restored the retina's insulin receptor activity and Akt signaling pathway [57,163,176]. Rosiglitazone is an antidiabetic drug that binds to peroxisome proliferator-activated receptor gamma (PPAR-γ), increasing insulin sensitivity [177]. Rosiglitazone treatment showed anti-apoptotic activity in the retinas of diabetic animals [178]. Sitagliptin is another antidiabetic drug used to treat DR in an experimental model. Sitagliptin treatment has demonstrated a protective effect by decreasing inflammation and apoptosis in the retina of diabetic rats [179,180].

3.4.2. Anti-hyperlipidemia agents

Fibrates activate peroxisome proliferator-activated receptor alpha (PPAR-α), effectively delaying the progression of DR [181,182]. Fenofibrate is primarily used for hypercholesterolemia but is also reported to reduce apoptosis, oxidative stress, and inflammation in diabetic retinas [183]. Fenofibrate decreased the expression of COX-2, an essential inflammatory molecule, in rat retinal endothelial cells (RRECs) cultured in a high glucose media [184]. Fenofibrate also inhibited NF-κB activity in human retinal pigment epithelium cells (ARPE-19) and reduced the expression of

IL-6, IL-8, and MCP-1 when cultured in high glucose media and IL-1β [185]. In the type 2 diabetic mice model, treatment with fenofibrate reduced gliosis and improved retinal neuronal function [186].

3.4.3. Anti-inflammatory agents

Infliximab is an anti-inflammatory medication that inhibits TNF and causes disruption of pro-inflammatory cascade signaling [187]. Treatment of patients with diabetic macular edema by infliximab improved visual acuity [188]. Salicylates are another example of an anti-inflammatory treatment that showed a reduction in the incidence of DR in patients who used salicylates for rheumatoid arthritis [189]. Minocycline is another medication with anti-inflammatory properties primarily used as an antimicrobial agent [190]. Minocycline reduced inflammation and caspase-3 activity in the retina of diabetic rats [191].

3.4.4. Neuroprotective agents

Treatment with nerve growth factor (NGF) prevented against loss of RGCs and Müller cells in STZ-diabetic rats [61]. NGF treatment also prevented pericyte loss and acellular capillaries [61]. PEDF is an essential factor in the retina that protects the retina against oxidative damage and glutamate toxicity [192]. Treatment of diabetic rats with PEDF showed a protective effect against early diabetic retinopathy by improving retinal neuronal function, reducing oxidative stress, and downregulating glial cell activation [193]. While diabetic rats showed a reduction of BDNF in RGCs, amacrine cells, and Müller cells [162], the treatment with BDNF reduced the damage to amacrine cells and ganglion cells [162,194]. VEGF is also an essential neurotrophic factor in the retina that maintains retinal neurons [84]. VEGF inhibition in the normal retina led to a noticeable

loss of ganglion cells [195], while VEGF treatment rescued retinal neurons in mice from neurotoxicity [196].

3.4.5. Antioxidants

Antioxidants are capable of preventing neurodegeneration in the diabetic retina by reducing oxidative stress. Different antioxidants have been studied in diabetic animal models. For example, the lutein-supplemented diet protected the retinas of diabetic mice against the increase of ROS and decreased diabetes-induced apoptosis [197]. Lutein also suppresses visual impairment caused by diabetes and protects against retinal histological changes [197]. In addition, stobadine and vitamin E are antioxidants that protect against lipid peroxidation in the retinas of diabetic rats [198].

Cardiolipin is a phospholipid present in the inner membrane of mitochondria [199]. It plays an essential role in energy metabolism and production [199], and it is also critical in the mitochondrial apoptosis [199]. Alterations in cardiolipin have been found in diabetes [199]. There is some evidence that cardiolipin antibody is increased in high-risk patients with PDR [200]. However, a new drug known as MTP-131 targets mitochondrial cardiolipin and promotes an efficient electron transfer [201]. Early treatment with MTP-131 in mouse models of type 1 and type 2 diabetes, via systemic or eye drop during the visual decline phase prevented further reduction and improved visual function without changing glucose clearance or hyperglycemia or body weight [201].

4. Polyamines and neuroprotection

Polyamines, including spermine, spermidine, and putrescine, are essential for cellular functions such as proliferation, differentiation, and apoptosis. Polyamines also maintain DNA stability [202].

Polyamines also regulate channel activity, including potassium channels (Kir channels) and inotropic glutamate receptors [203,204].

4.1. Polyamine synthesis and oxidation

Polyamine metabolic pathways play a crucial role in regulating and maintaining polyamine homeostasis. The metabolism of polyamines is regulated by the coordinated actions of multiple enzymes (Figure 1.2). Spermine and spermidine are converted to spermidine and putrescine, respectively, through back-conversion by SSAT (spermidine/spermine N1-acetyltransferase) to N1-acetylated polyamines. N1-acetylated polyamines are oxidized by polyamine oxidase (PAO) to produce spermidine and putrescine, respectively [205]. In addition to the PAO oxidation pathway, spermine also can be directly oxidized by spermine oxidase (SMOX) to spermidine. PAO and SMOX regulate polyamine oxidation in mammalian cells, and the activation of these enzymes

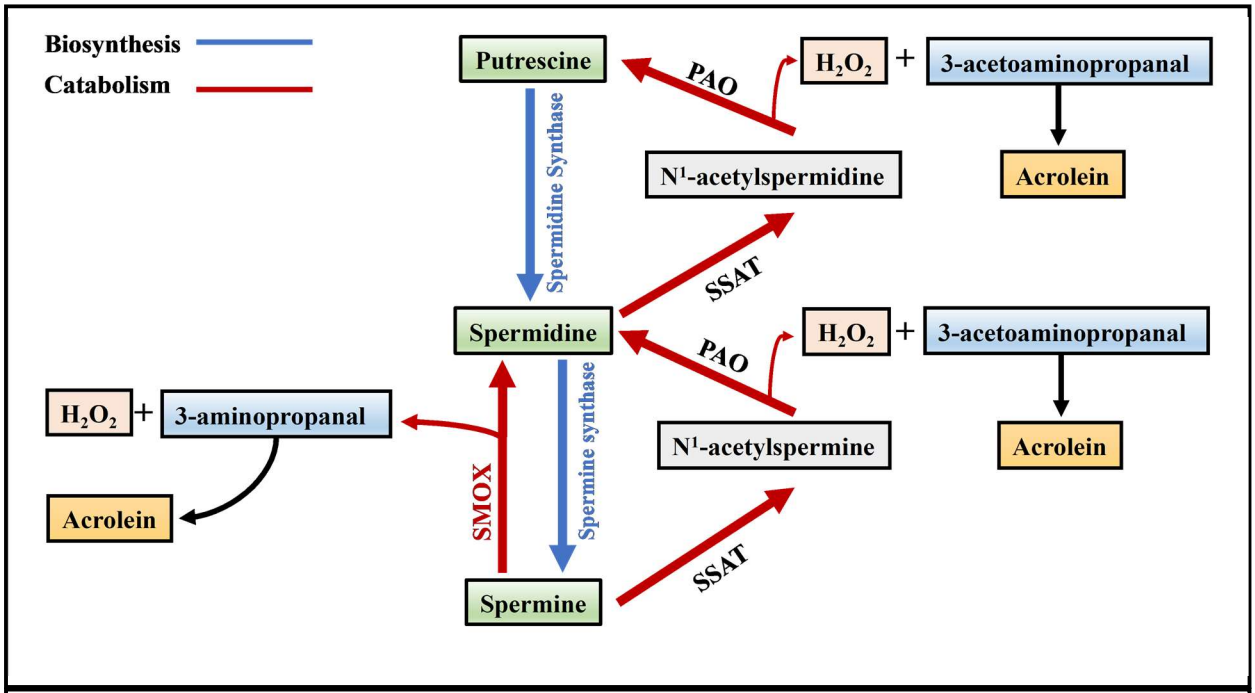


Figure 1.2. Diagrammatic representation of polyamines regulation by biosynthesis and oxidation enzymes. PAO: polyamine oxidase; SMOX: spermine oxidase; SSAT: spermidine/spermine N1 acetyltransferase [1].

causes the release of H_2O_2 and acrolein [205]. While SMOX is a highly inducible enzyme, PAO is constitutively expressed [206].

4.2. Spermine oxidase and neurodegeneration

Dysregulation of polyamines metabolism is involved in neurodegenerative diseases such as ischemic brain injury [207], traumatic brain injury [208], and Parkinson's disease [209]. The overexpression of SMOX causes the activation of glial and microglial cells and an increase in H_2O_2 production in the neocortex in response to excitotoxic injury [210]. On the other hand, the downregulation of SMOX in a model of ischemic injury reduced the infarcted volume and neurological deficit, in addition to a reduction in neuronal apoptosis [211]. SMOX downregulation also reduced microglia activation and reduced the release of IL-6 and TNF- α cytokines after stroke in rats [211]. Also, pharmacological or genetic inhibition of SMOX showed a reduction in H. pylori-induced inflammation and DNA damage in mice [212,213]. Oxidation of polyamines causes the release of H_2O_2 and reactive aldehydes (that rapidly get converted to acrolein) as byproducts. Acrolein is highly toxic and a potent mediator of oxidative damage. There are different endogenous sources of acrolein, but the primary source is polyamine oxidation [214]. Acrolein has an important role in the progression of diseases such as atherosclerosis [215], cardiovascular disease [216], pulmonary inflammation [217], and kidney disease [218]. Acrolein is also involved in neurodegenerative diseases such as Alzheimer's [219], multiple sclerosis [220], brain infarction [221], and spinal cord injury [222]. Acrolein exposure may cause cognitive problems and lead to severe neurocognitive effects on humans [223]. Acrolein's harmful effects are mediated through multiple mechanisms, including DNA damage, inflammation, ROS formation, protein adduction, membrane disruption, endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis

(Figure 1.3) [224-226]. In vitro studies revealed that injury induced by acrolein was more toxic than ROS [227,228]. Acrolein also has been shown to induce inflammation in mouse BV2 microglial cells and function as a neurotoxin causing increased cell death in a rat model [118,229].

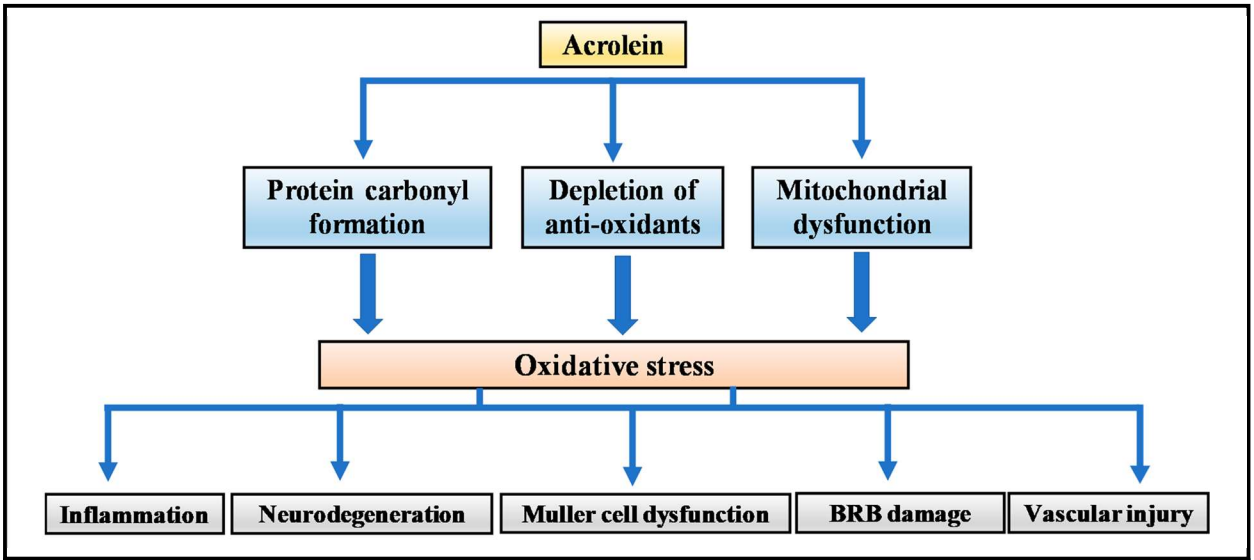


Figure 1.3. Schematic representation of the potential mechanism of action of acrolein in diabetic retinopathy [1].

Studies have reported acrolein's association in type 1 and type 2 diabetes. Elevated urinary level of conjugated acrolein is reported in patients with type 1 and type 2 diabetes [230,231]. These clinical studies illustrated that a high urinary acrolein level is linked to the diabetic stage and significantly correlated to the glycemic status [230,231]. In a group of younger type 1 diabetic patients, the urine level of conjugated acrolein and other oxidative stress markers (pentosidine, pyrraline, and 8-hydroxy-20 -deoxyguanosine), was shown to be significantly elevated than healthy subjects [230]. The study performed by Daimon et al. and colleagues [231] in type 2 diabetic patients exhibited significantly increased acrolein adduct, pentosidine, and pyrraline in urine samples. Furthermore, glycemic control parameters, such as fasting plasma glucose and HbA1c (hemoglobin A1c, the glycated hemoglobin level), were observed to correlate with these markers urinary levels. The acrolein adduct level was higher in smokers with diabetes than in diabetic non-

smokers and non-diabetic groups [231]. In addition, acrolein protein adducts are associated with diabetes-related complications such as diabetic nephropathy [232] and diabetic retinopathy [233].

4.3. Inhibitors of spermine oxidase

N1N4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527) is a selective irreversible inhibitor of polyamine oxidases [234]. Previous studies have reported that SMOX inhibition by treatment with MDL 72527 showed neuroprotective effects in ischemic brain injury in rats by reducing edema formation, ischemic injury, neurological deficit, neuronal apoptosis, and inflammation volume [207,235]. MDL 72527 treatment was also neuroprotective against edema and necrotic formation in rats after traumatic brain injury [208]. Treatment with MDL 72527 also reduced the effect of inflammation-induced carcinogenesis in the model of H. pylori infection [213].

A previous study from our lab has shown that MDL 72527 treatment reduced oxidative stress and retinal neurodegeneration in the model of oxygen-induced retinopathy (OIR) [236]. Also, treatment with MDL 72527 in the OIR model protected the retina against vascular injury, inflammation, and glial activation [237]. In the excitotoxic retina, the number of apoptotic neurons was increased, and the number of RGCs was decreased, but the treatment with MDL 72527 protected retinal neurons against these changes [238].

Aminoguanidine is a specific and effective inhibitor of diamine oxidase (DAO). However, it decreased PAO activity in the ischemic brain and reduced the formation of 3-aminopropanal, which is a result of polyamine oxidation and converted spontaneously to acrolein (Figure 1.2) [239,240]. Aminoguanidine treatment also reduced the infarction volume in rats after ischemic injury [240,241]. Neuronal cytotoxicity was also reduced in response to aminoguanidine treatment, but it did not protect neuronal cells against NMDA excitotoxicity injury [240]. Chloroquine is an

antimalaria medication, but it also has the ability to reduce PAO activity [240]. Chloroquine also reduced 3-aminopropanal formation and neuronal cytotoxicity after ischemic brain injury [240].

MDL 72527 is a common inhibitor for both polyamine oxidases, SMOX and APAO. Bis(ethyl)norspermine (BENSpm) and N1-ethyl- N11-(cyclopropyl)-methyl-4,8-diazaundecane (CPENSpm) are two spermine analogs that have the ability to inhibit SMO. However, these two inhibitors have a poor affinity for the enzyme [242,243]. None of the inhibitors studied so far possesses the desired affinity and specificity to be used as a selective inhibitor. Therefore, searching for specific SMOX inhibitors is still an important open issue.

4.4. Spermine oxidase and diabetic retinopathy

Even though several studies have addressed the critical involvement of polyamine oxidation in neurodegenerative diseases, not much information is available in the field of diabetic retinopathy. A clinical study reported that the polyamine level was altered in vitreous samples from patients with proliferative DR [244]. Dysregulation in the polyamine pathway is involved in the pathogenesis of diabetes and diabetic retinopathy [48,245,246]. A previous study has shown that SMOX was localized in glial cells of fibrovascular tissue from patients with PDR [246]. Study from our lab indicates that, in diabetic mice, SMOX is highly expressed within GCL, OPL, and to some extent in INL [48]. The increase in SMOX in the retinas of diabetic mice was associated with a reduction in the number of RGCs, a reduction in total retinal thickness and ganglion cells complex (GCC) thickness, and a decline in retinal function [48]. In addition, studies from our laboratory have shown that polyamine oxidation mediates neuronal and vascular damage in the retina in OIR and NMDA-excitotoxicity-induced retina [236-238].

Acrolein, which results from polyamine oxidation, was elevated in urine in patients with T1D and T2D [230,231]. These clinical studies illustrated that a high urinary acrolein level is linked to the diabetic stage and significantly correlated to the glycemic status. Acrolein protein adducts are associated with diabetes-related complications such as diabetic nephropathy [232] and diabetic retinopathy [233]. Acrolein, which results from polyamine oxidation, was increased in the diabetic retina, and that increase is associated with a reduction in retinal function [48,107]. When diabetic mice were treated with MDL 72527, retinas expressed less conjugated acrolein [48]. The in-vitro experiment showed that Müller cells generated more conjugated acrolein under hypoxia conditions, but when cells were treated with polyamine oxidase inhibitor (MDL 72527), the formation of H_2O_2 and acrolein significantly reduced [246]. The accumulation of acrolein in Müller cells might contribute to Müller cells dysfunction in the early stages of the disease in animal models because it has been seen that it was associated with a rise in GFAP expression and reduction in K^+ channel in the retina of diabetic rats [107]. The increase of acrolein in the retina of diabetic rats was also associated with an increase in RAGE expression, which activates microglia that causes an increase in inflammatory molecules IL-1β, CCL2, and ICAM-1 [107].

5. Research Objectives

Objective 1: While the pharmacological inhibition of SMOX reduced retinal neuronal damage, the exact mechanisms by which SMOX causes retinal neurodegeneration is not completely understood. To better understand the role of SMOX in retinal neuronal damage, a mechanistic model for retinal neurodegeneration will be used to study the underlying molecular mechanisms by SMOX activation in the retina, including inflammation and oxidative stress.

Objective 2: Several studies have evaluated the role of SMOX inhibition on retinal injuries; however, the effects of long-term inhibition of SMOX in DR have not studied. To study this objective, long-term treatment with a pharmacological inhibitor of SMOX will be performed on an experimental model for type-1 diabetes.

6. Specific aims

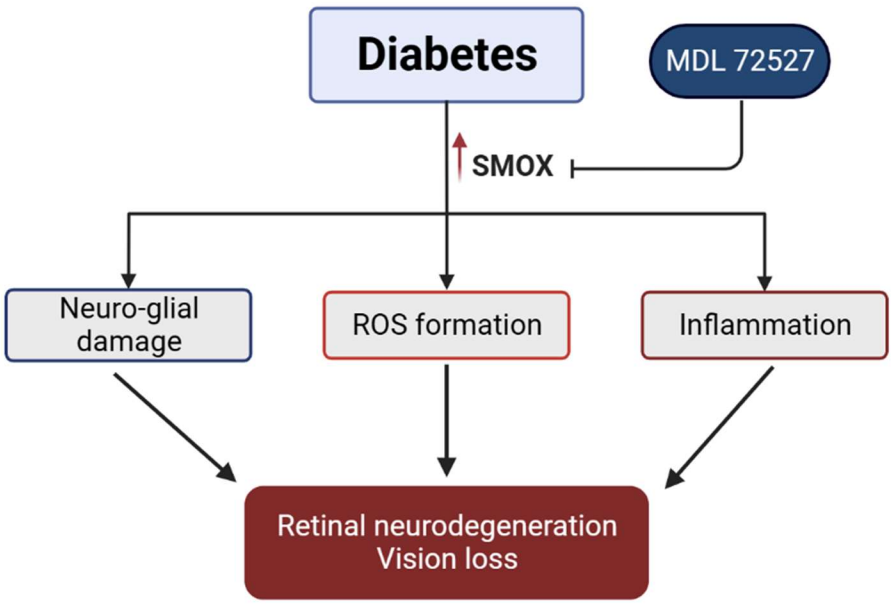
Aim 1: Test the hypothesis that SMOX inhibition using MDL 72527 reduces inflammation and oxidative damage in the retina.

Sub aim 1: Utilizing the retinal excitotoxicity model and SMOX inhibitor (MDL 72527), this study investigates the impact of SMOX inhibition on the status of retinal microglia/macrophage, cytokine release, acrolein conjugates, and the signaling pathways involved.

Sub aim 2: Study the effect of acrolein (a product of SMOX activity) on mediating retinal inflammation. Using the C8-B4 cells (microglia/macrophage cell line), this study evaluates the effect of acrolein treatment on morphological changes and ROS formation.

Aim 2: Test the hypothesis that long-term inhibition of SMOX using MDL 72527 will suppress neuro-glial damage and visual acuity in diabetic mice. This aim utilizes streptozotocin (STZ)-induced diabetic mice model of DR and MDL 72527 treatment for 24 weeks following the induction of diabetes.

Figure (1.4) shows the proposed mechanism of SMOX activation and neurodegeneration in diabetic retina.



1.4. Proposed role of SMOX in diabetic retinopathy. A diagrammatic representation of the proposed molecular mechanism of SMOX mediated retinal neurodegeneration and vision loss in diabetes. Created in BioRender.com

7. Significance and innovation

The vision loss associated with DR affects patients' quality of life and has negative social and psychological effects. The unavailability of effective treatments to prevent the onset or progression of DR is a major clinical problem. Since the approved treatments are only for late-stage diabetic retinopathy, they only treat vascular abnormality but not neuronal changes. Therefore, it is important to find better and early interventions that prevent or delay vision loss in diabetic patients. Neurodegeneration is recognized as an early event in DR, and studies from our laboratory show that inhibition of SMOX reduced diabetes-induced neurodegeneration in the retina. Supporting evidences indicate that the inhibition of SMOX offers anti-inflammatory and neuroprotective effects. The treatment with MDL 72527 could be a good candidate for treating neurodegeneration and neuroinflammation and ultimately reducing vision loss in diabetic patients.

CHAPTER II: SPECIFIC AIM 1

Pharmacological Inhibition of Spermine Oxidase Suppresses Excitotoxicity Induced Neuroinflammation in Mouse Retina[1]

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[1] Moaddey Alfarhan, Fang Liu, Shengshuai Shan, Prahalathan Pichavaram, Payaningal R. Somanath, and S. Priya Narayanan. Published in *International Journal of Molecular Sciences*. 2022; 23(4):2133

Abstract

Polyamine oxidation plays a major role in neurodegenerative diseases. Previous studies from our laboratory demonstrated that spermine oxidase (SMOX, a member of the polyamine oxidase family) inhibition using MDL 72527 reduced neurodegeneration in models of retinal excitotoxicity and diabetic retinopathy. However, the mechanisms behind the neuroprotection offered by SMOX inhibition are not completely studied. Utilizing the experimental model of retinal excitotoxicity, the present study determined the impact of SMOX blockade on retinal neuroinflammation. Our results demonstrated an upregulation in the number of cells positive for Iba-1 (ionized calcium-binding adaptor molecule 1), CD (Cluster Differentiation) 68, and CD16/32 in excitotoxicity-induced retinas, while MDL 72527 treatment reduced these changes. MDL 72527 treatment increased the number of cells (anti-inflammatory) positive for Arginase1 and CD206. When retinal excitotoxicity upregulated several pro-inflammatory genes, MDL 72527 treatment reduced many of them, and increased anti-inflammatory genes. Furthermore, SMOX inhibition upregulated antioxidant signaling (indicated by elevated Nrf2 and HO-1 levels) and reduced protein-conjugated acrolein in excitotoxic retinas. In vitro studies using C8-B4 cells showed changes in cellular morphology and increased reactive oxygen species formation in response to acrolein (a product of SMOX activity) treatment. Overall, our findings indicate that the inhibition SMOX pathway reduced neuroinflammation and upregulated antioxidant signaling in the retina.

1. Introduction

Neuroinflammation is an important cause in the development of neurodegenerative diseases [247,248]. Long-term inflammation exposure causes cellular damage [249]. Studies show that inflammation has an essential role in the pathogenesis of several ocular diseases, including age-related macular degeneration (AMD) [250], diabetic retinopathy [251], and retinitis pigmentosa [252]. Injured neurons are capable of releasing damage-associated molecular patterns (DAMPs), and these DAMPs can bind to their receptors on microglial cells and cause activation of microglia [253]. Activation of microglia, which is the primary resident immune cell in the brain and retina, results in the release of inflammatory mediators such as cytokines, reactive oxygen species (ROS), and nitric oxide (NO) [254]. Retinal excitotoxicity is suggested as a major mechanism of neurodegeneration and experimental models of excitotoxicity are extensively used for studying retinal ganglion cells (RGCs) damage and dysfunction in ocular diseases including optic neuropathy [255,256], glaucoma [84,257-259], and diabetic retinopathy [84,257].

Polyamines including spermine, spermidine, and putrescine are important for cellular functions such as proliferation, differentiation, and apoptosis [206]. N1-acetylpolyamine oxidase (APAO) and spermine oxidase (SMOX) regulate polyamine catabolism, but the activation of these enzymes causes the release of H_2O_2 and acrolein [205]. While SMOX is a highly inducible enzyme, APAO is constitutively expressed [206]. Acrolein has shown to induce inflammation in mouse BV2 microglial cells and function as a neurotoxin causing increased cell death in a rat model [229,260]. N1N4-bis (2, 3-butadienyl)-1,4-butane diamine (MDL 72527) is a selective-irreversible inhibitor for APAO and SMOX [234,261,262]. Previous studies have reported that MDL 72527 treatment showed neuroprotective effects in ischemic brain injury in rats by reducing edema formation and reduction in ischemic injury volume [207,235]. Treatment with MDL 72527 was also

neuroprotective against edema formation and necrotic formation in rats after traumatic brain injury [208].

Earlier studies from our laboratory have demonstrated that MDL 72527 treatment reduced retinal neurovascular damages in models of hyperoxia [236] and retinal excitotoxicity [238]. Studies performed on the experimental model of DR demonstrated that the inhibition of SMOX by treatment with MDL 72527 improved RGC survival, retinal structure, and function in diabetic mice [48]. Our previous study has also reported an increase in the expression of SMOX during retinal excitotoxicity and the degeneration of neurons, while MDL 72527 treatment improved neuronal survival [238]. However, the mechanisms behind the neuroprotective effects of SMOX blockade are not studied. Neuroinflammation is considered a major cause of neurodegeneration, and hence using a mouse model of retinal excitotoxicity, the present study was undertaken to investigate whether the inhibition of SMOX by MDL 72527 can ameliorate neuroinflammation.

2. Materials and Methods

2.1 Animals

All the procedures using animals were performed by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee (Animal Welfare Assurance no. A3307–01) adhered to the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised July 2018). The protocols are approved by the Institutional Animal Care and Use Committee of the Augusta University (2016-0823) and the Charlie Norwood VA Medical Center, Augusta (18-11-110). Wild-type male C57BL6J mice (8–10 weeks, Jackson

Laboratories, Bar Harbor, ME) were used in this study, and efforts were made to assure the minimum possible suffering during experimental procedures.

2.2 Induction of retinal excitotoxicity

Retinal excitotoxicity is suggested as a major mechanism of neurodegeneration in DR, and experimental models of excitotoxicity are extensively used for studying diabetes-induced retinal neurodegeneration [68,69]. Retinal excitotoxicity was induced according to the method established in our laboratory [238]. Mice were anesthetized with 73 mg/kg ketamine hydrochloride and 7.3mg/kg xylazine hydrochloride intraperitoneally. Pupils were dilated with 1% tropicamide (Akorn, Lake Forest, IL). One drop of proparacaine hydrochloride (Akorn) was applied to the cornea as topical anesthesia. NMDA (N-Methyl- D-Aspartate, (Sigma), 20 nmoles/eye, 1µl, dissolved in saline) was injected intravitreally into the right eye using a beveled 35 G needle (NF35BV-2, World Precision Instruments, FL) connected with an SGE Syringe (World Precision Instruments, FL). The needle was moved out slowly after maintaining for 30 seconds. Antibiotic ointment was applied to prevent infection. NMLA (N-Methyl L Aspartate, 20 nmoles/left eye) was used as a control.

2.3 MDL 72527 treatment

In previous studies from our lab, with short-term treatments, 40 mg/kg/day was more effective than lower doses (10 mg/kg and 20 mg/kg); and the high dosage of MDL 72527 (100 mg/kg) worsened the injury [237,238]. Some studies used MDL 72527 in drinking water (0.05% w/v) or within the animal's diet [263-265]. However, we preferred injecting the drug into mice to ensure that all mice received the exact dose. Intravitreal injunctions of MDL 72527 were avoided because

we were looking for less invasive treatments. Also, avoid some side effects of intravitreal injections as inflammation or infection of the eye. Treatment with polyamine oxidase inhibitor MDL 72527 (Sigma, 40 mg/kg/day) in 0.9% saline was performed as described [238]. Treatment started one day before NMDA or NMLA injection and continued until the end of the respective experiments (varied from three to seven days). Normal saline was used as vehicle treatment.

2.4 Immunostaining of retinal flat-mount and quantification of Iba-1 positive cells

Immunostaining of retinal flat mounts was performed as described previously [237,266]. Eyes were removed and fixed in 4% paraformaldehyde overnight at 4°C. After removing the cornea, sclera, lens, vitreous, and hyaloid vessels, four radial incisions were made to allow flattening of the retina. Retinas were permeabilized with 10% Triton X-100 for 20 minutes and blocked with 10% normal goat serum containing 1% BSA and 0.1% Triton X-100 for 1 h at room temperature. Retinas were stained with Iba-1 (1:500, Wako, Richmond, VA) at 37 °C for 2 h, followed by secondary antibody Alexa-Fluor 555 goat anti-rabbit (1:500) (Life Technologies, Carlsbad, CA) overnight at 4°C. Retinas were washed three times in PBS and flat-mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Flat mounts were examined using a confocal microscope (Zeiss LSM 510 META, Thornwood, NY) and serial images were acquired. Quantification of Iba-1 positive cells (with activated morphology) GCL layer was performed using NIH ImageJ software as described in the previous reports from our laboratory [238,267,268]. Images (non-overlapping fields from the optic nerve to the edge) were collected from each quadrant of the flat-mounted retina at the mid periphery (defined as halfway between the optic nerve head and the outer periphery). Five serial images (1 μm apart) of Iba-1 positive cells were taken from each region and

merged to get a projection image for quantification. Cells showing activated morphology were selected manually (using point tool) and quantified using the analyze particle function of ImageJ.

2.5 Western Blotting

Retinal tissues were collected, snap-frozen immediately, and stored at -80 C until used. Samples were homogenized in 1X RIPA lysis buffer (Millipore, Billerica, MA) containing protease inhibitor cocktail (Thermo Scientific™) and phosphatase inhibitor cocktail (Thermo Scientific™). Protein concentration was estimated using BCA Protein Assay Kit (Thermo Scientific™). Approximately 20-30 µg of protein samples were separated on 8-12% SDS-PAGE gel, transferred to PVDF membrane, and blocked in 5% non-fat dry milk in 1X Tris-buffered saline with 0.1% Tween (TBST) for 1 hour. Membranes were incubated overnight at 4°C with respective primary antibodies (Table 1). The membranes were then washed with TBST 3 times and incubated with respective secondary antibodies (anti-rabbit or anti-mouse HRP-conjugated secondary antibody,

Antibody	Catalog number	Source	Dilution	Experiment
Iba-1	019-19741	Wako	1:500	Immunostaining
Iba-1	17198	Cell Signaling Technology	1:500	Western blot
Arginase-1	Abs535	EMD Millipore	1:200	Immunostaining
CD68	ab125212	Abcam	1:500	Immunostaining
CD16/32	553142	BD Biosciences	1:500	Immunostaining
CD206	ab64693	Abcam	1:500	Immunostaining
F4/80	ab6640	Abcam	1:100	Immunostaining
Nrf2	12721	Cell Signaling Technology	1:1000	Western blot
HO-1	43966	Cell Signaling Technology	1:1000	Western blot
Conjugated-acrolein	ab48501	Abcam	1:1000	Western blot
β-Actin	A1978	Sigma-Aldrich	1:10000	Western blot

Table 2.1. List of antibodies used in the study

Table 1) for 1 hour at room temperature. The membranes were washed with TBST 3 times. Protein bands were detected using enhanced chemiluminescence (ECL) (Thermo Fisher). Band intensities were quantified using ImageJ software and normalized to beta-actin as the loading control.

2.6 Immunofluorescence staining and quantification of cells on retinal sections

Immunostaining of retinal cryostat sections was performed as per the methods established in our laboratory [48,266,269]. Briefly, eyes were removed and fixed in 4% paraformaldehyde overnight at 4 °C and cryoprotected in 30% sucrose for 24 hours. Cryostat sections (10 µm) obtained on glass slides were permeabilized in 0.1% Triton X-100 (30 min), followed by blocking in 10% normal donkey serum for 1 h at room temperature. Sections were incubated with primary antibodies (Table 1) overnight at 4 °C. Next day, sections were washed 3 times with PBS and then incubated with Fluorescein-conjugated secondary antibodies (Table 1) for 2 hours at room temperature. The sections were then washed 3 times in PBS and covered with a mounting medium with DAPI (Vectashield; Vector Laboratories, Burlingame, CA). Images were obtained using a Zeiss (Thornwood, NY) Axioplan Imager microscope and Zeiss Axiovision 4.8.2 software. Images (40X) were taken at 500 µm from the optic nerve and the number of cells positive for CD68 or CD16/32 was counted manually. For the quantification of A1 or CD206 positive cells, counting was performed on retinal sections manually from the optic nerve head to the periphery. A minimum of three sections (20 µm apart) were used per animal and a minimum of five animals were used per group.

2.7 RNA isolation and quantitative RT-PCR

The total mouse retina was homogenized by a Micro-Tube homogenizer (F65100-0000, SP BEL-ART) using QIAzol Lysis Reagent (79306, QIAGEN). Total RNA from homogenized retinal tissues was extracted using miRNeasy mini kit (217084, Qiagen). The concentration of RNA was measured using a Nanodrop Lite Spectrophotometer (ND-LITE-PR, Thermo Fisher Scientific). Around 500 ng of total RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems). Quantitative PCR was carried out by StepOnePlus™ Real-Time PCR System (4376600, Applied Biosystems) using Power SYBR Green Master Mix (4309155, Applied Biosystems). Sequences of primers used in this study are

GENE NAME	FORWARD PRIMER	REVERSE PRIMER
TNFα	GGTCCCCAAAGGGATGAGAA	TGAGGGTCTGGGCCATAGAA
IL1β	CCAAGCAACGACAAAATACC	GTTGAAGACAAACCGTTTTTCC
IL10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
MCP-1	GGCTCAGCCAGATGCAGTTAA	CCTACTCATTGGGATCATCTTGCT
IL6	AGACAAAGCCAGAGTCCTTCAG	TGCCGAGTAGATCTCAAAGTGA
IL18	TCAAAGTGCCAGTGAACCCC	GGTCACAGCCAGTCCTCTTAC
COX2	AAGCCAACATGATTGTTGTGAA	CGGCAGCAGTCACATACTTA
IL21	ATCCTGAACTTCTATCAGCTCCAC	GCATTTAGCTATGTGCTTCTGTTTC
CCL5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
TGFβ	CCCGAAGCGGACTACTATGC	ATAGATGGCGTTGTTGCGGT
IL12	CTCAGGATCGCTATTACAATTCCTC	TTCCAACGTTGCATCCTAGGATC
IL17	CTCCAGAAGGCCCTCAGACTAC	AGCTTTCCCTCCGCATTGACACAG
CCL3	TTCTCTGTACCATGACACTCTGC	CGTGGAATCTTCCGGCTGTAG
IL4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
HPRT	GAAAGACTTGCTCGAGATGTCATG	CACACAGAGGGCCACAATGT

Table 2.2. Primer sequences used in the study

listed in Table 2. Data were normalized to hypoxanthine phosphoribosyl transferase (HPRT), and the fold change between levels of different transcripts was calculated by the ΔΔCT method.

2.8 Cell culture and conjugated acrolein treatment and immunocytochemistry

C8-B4 cells (ATCC) were kindly provided by Dr. Susan Fagan (University of Georgia), were cultured in DMEM high glucose medium (Gibco) and 10% FBS (Atlantic biologics), 100 I.U. /mL penicillin and 100 (µg/mL) streptomycin (ATCC). Cells were cultured at a density of 15,000 cells/well in chamber slide (Thermo Fisher) with complete medium, and treated with 25 µg/ml acrolein-BSA conjugate (StressMarq Biosciences) in complete medium for 6 hours. Following the incubation, the culture media was removed, and cells were washed with 1× PBS and fixed with 4% paraformaldehyde for 10 min and subjected to immunocytochemistry according to the method standardized in our laboratory [269,270]. This was followed by PBS wash, and the chamber slides were stored in humidified containers at 4 °C. Permeabilization of the cells was performed using 0.1% Triton X-100 in PBS (5 min), followed by PBS wash, and blocking was achieved by using 10% donkey serum at room temperature for 1 h. Cells were washed and incubated with respective primary antibodies (Table 1) overnight. The next day, the cells were washed with PBS and incubated with appropriate secondary antibodies for 2 h. The chamber slides were washed, and the chambers were separated from the glass slide. Cells were covered with a coverslip using a mounting medium containing DAPI and stored at 4 °C. Images were taken using a confocal microscope (LSM 780; Carl Zeiss, Thornwood, NY) available at the Augusta University imaging core facility.

2.9 Measurement of ROS generation using CM-H2DCFDA assay

This assay was performed using CM-H2DCFDA (C6827, Thermo Fisher) following the manufacturer's protocol. After appropriate treatment, C8-B4 cells were washed with pre-warmed PBS (37°C) and incubated with ROS-sensitive probe CM-H2DCFDA (40 μM) at room temperature in the dark for 30 minutes. Cells were washed carefully with warm PBS and covered with a mounting medium with DAPI (Vectashield; Vector Laboratories, Burlingame, CA). Images were taken immediately by using the FITC channel. The intensity was quantified by using NIH ImageJ software.

2.10 Data Analysis

GraphPad Prism 9 was used for statistical analysis. Results are presented as mean ±SD. Statistical analysis was performed using one-way ANOVA followed by the Tukey test for multiple comparisons. Student's t-test is used when two independent groups are compared.

3. Results

3.1 SMOX inhibition using MDL 72527 reduced Iba-1 positive cells in the excitotoxic retina

Our previous study has shown that SMOX blockade by MDL 72527 significantly improved neuronal survival in the excitotoxic retinas [238]. In the present study, we first investigated the status of the inflammatory cells positive for Iba-1 (ionized calcium-binding adaptor molecule 1), a marker specific for microglia/macrophages. Immunofluorescence staining of retinal flatmounts demonstrated an increase in the number of Iba-1 positive cells presenting activated morphology in the retinas from mice treated with NMDA (7 days post-injury) compared to their NMLA controls (Figure 2.1 A&B). In response to SMOX inhibition by treatment with MDL 72527, a marked

reduction of Iba-1 positive microglia/macrophage cells was observed in comparison with the NMDA group (Figure 2.1 B&C). MDL 72527 treatment did not result in any noticeable changes in the control retina (Figure 2.1 D). Quantification studies indicated a significant increase in cells with activated morphology while MDL 72527 treatment significantly reduced this effect (Figure 2.1 E). Furthermore, our immunoblot studies using retinal lysates showed that the upregulation in the expression of Iba-1 protein level in NMDA retinas was significantly reduced in response to MDL 72527 treatment (Fig 2.1 F&G). Altogether, these results suggest the potential of MDL 72527 treatment in reducing inflammation in excitotoxic retinas.

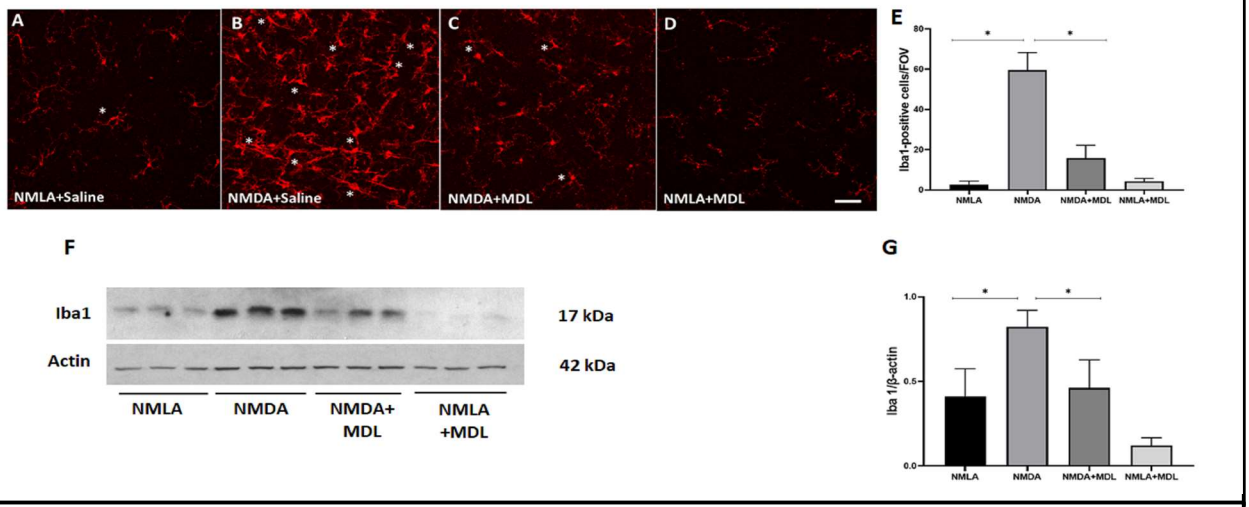

Figure 2.1. Treatment with MDL 72527 reduced Iba-1 positive cells with activated morphology in the excitotoxic retina. A-D) Immunofluorescence staining of retinal flat mounts using Iba-1 antibody and representative confocal images presented. Asterisks indicate retinal microglia presenting activated morphology (7 days post-injury). E) Bar graph representing the quantification studies of Iba-1 positive cells with activated morphology (N=4-6 per group, *P<0.01). F&G) Results of Western blot studies showing the increased protein levels of Iba-1 in NMDA retinas, compared to NMLA controls. Iba-1 expression was reduced in response to MDL 72527 treatment. *P < 0.01, N=4-5 per group. Scale bar 50 µm. Results presented as Mean ± SD.

3.2 Impact of MDL 72527 treatment on the status of inflammatory cells in response to excitotoxicity

We next investigated whether SMOX inhibition using MDL 72527 affected different populations of inflammatory cells. Microglia/macrophages in the CNS have been reported to adopt distinctive phenotypes, including the classically activated (M1) state and the alternatively activated (M2) state in response to various stimulations [271]. Immunofluorescence staining of retinal cryostat sections (3 days post-injury) was performed using markers of M1/M2 phenotypes of microglia/macrophages. Our results show that the number of M1 phenotype cells positive for CD (Cluster of Differentiation) 68 and CD 16/32 were significantly increased in excitotoxicity-induced retinas (Figure 2.2 A&B and F&G). This effect was significantly reduced in the excitotoxic retinas in response to the treatment with MDL 72527 (Figure 2.2 C & H).

On the contrary, studies using M2 markers showed significant upregulation in the cells positive for Arginase (A) 1 and CD 206 in MDL 72527 treated NMDA retinas compared to vehicle treated group (Fig 2.2 L&M and Q&R). Our immunofluorescence studies indicated that the majority of the CD 206 positive cells are colocalized with cells positive for F4/80, a major marker of macrophages. MDL 72527 treatment did not cause any marked changes in the status of CD68 or CD16/32 positive cells in the control groups. The slight upregulation observed in A1 and CD 206 in NMLA control group in response to SMOX inhibition, however, was not significant. These results indicate that SMOX blockade is capable of reducing inflammatory cells in response to excitotoxicity.

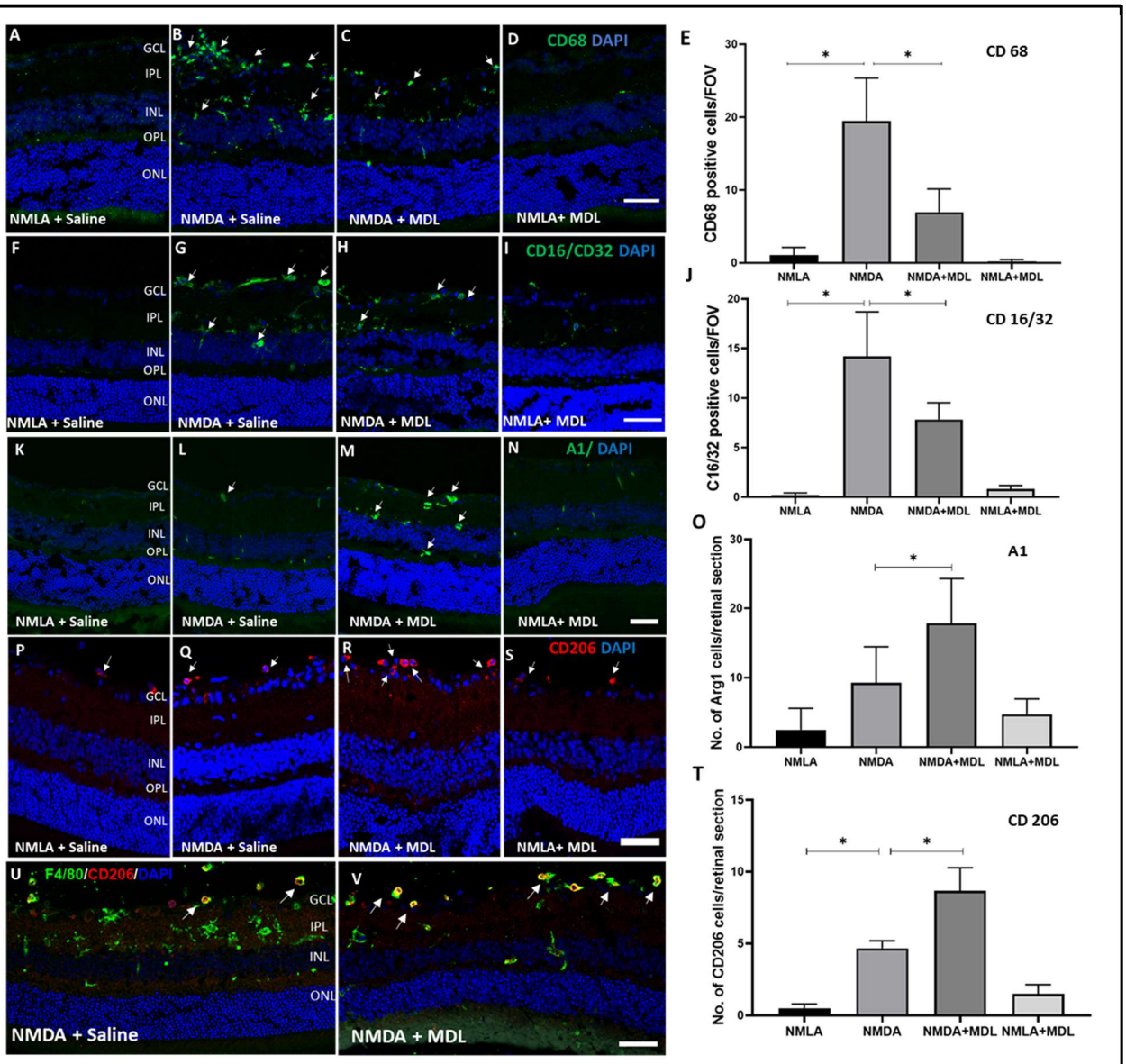

Figure 2.2. SMOX inhibition altered the level of M1 and M2 microglia/macrophage phenotypes in the excitotoxic retinas. Representative confocal images of retinal cryostat sections immunostained using CD 68 (A-D), CD 16/32 (F-I), A1 (K-N) and CD 206 (P-S). Bar graphs showing the quantification studies demonstrate that number of cells positive for M1 markers, CD 68 (E) and CD 16/32 (J) are upregulated in excitotoxic retinas while MDL 72527 significantly downregulated the changes. Quantification of data showed a significant upregulation in the number of cells positive for M2 markers, A1(O) and CD 206 (T) in the NMDA retinas, in response to MDL 72527 treatment. U&V) Confocal images showing the colocalization of CD 206 and F4/80 in excitotoxic retinas, represented by the arrows. Number of animals used N=3-6 per group. *P<0.05, 3 days post-injury. Scale bar 50 µm. Arrows indicate representative cells positive for the markers used. Results presented as Mean ± SD.

3.3 Effect of SMOX inhibition on pro- and anti-inflammatory cytokines

We next investigated the changes in the status of inflammatory molecules in relation to SMOX blockade (Figure 2.3). Analysis of mRNA levels using qRT-PCR at two different time points (3 and 5 days) following excitotoxic insult was performed (Figure 2.3 A&B). Our results indicated that, in response to MDL 72527 treatment, there is an early upregulation in the anti-inflammatory cytokines followed by downregulation of pro-inflammatory molecules in the excitotoxic retinas. Significant upregulation of many pro-inflammatory genes including interleukin (IL-) 1β, IL-21, C-C motif chemokine ligand (CCL) 5, CCL3, cyclooxygenase 2 (COX2), tumor necrosis factor-alpha (TNF-α), and chemoattractant protein-1 (MCP-1) in NMDA retinas was observed at both time points as compared to the control retinas. As evidenced in Figure 2.3A, 3 days post-injury, MDL 72527 treatment significantly upregulated many anti-inflammatory genes IL-4, IL-10, IL-13, and transforming growth factor β (TGF-β) in the excitotoxic retinas, CCL5 is the only pro-inflammatory gene that showed a significant downregulation. MDL 72527 treatment did not reduce the mRNA levels of other pro-inflammatory molecules (Fig 2.3 A). Interestingly, on day 5 following excitotoxicity, SMOX inhibition demonstrated reduced levels of several of the inflammatory molecules such as IL-1β, CCL3, IL-21, and TNF-α levels in NMDA retinas. However, the levels of anti-inflammatory genes were similar across the two NMDA-treated groups at this time point (Figure 2.3 B). However, the decrease observed in the levels of IL-6, COX2, CCL5, and MCP-1 was not statistically significant (Figure 2.3 B). These results further suggest the potential of SMOX blockade in reducing the inflammatory signals in response to retinal excitotoxicity.

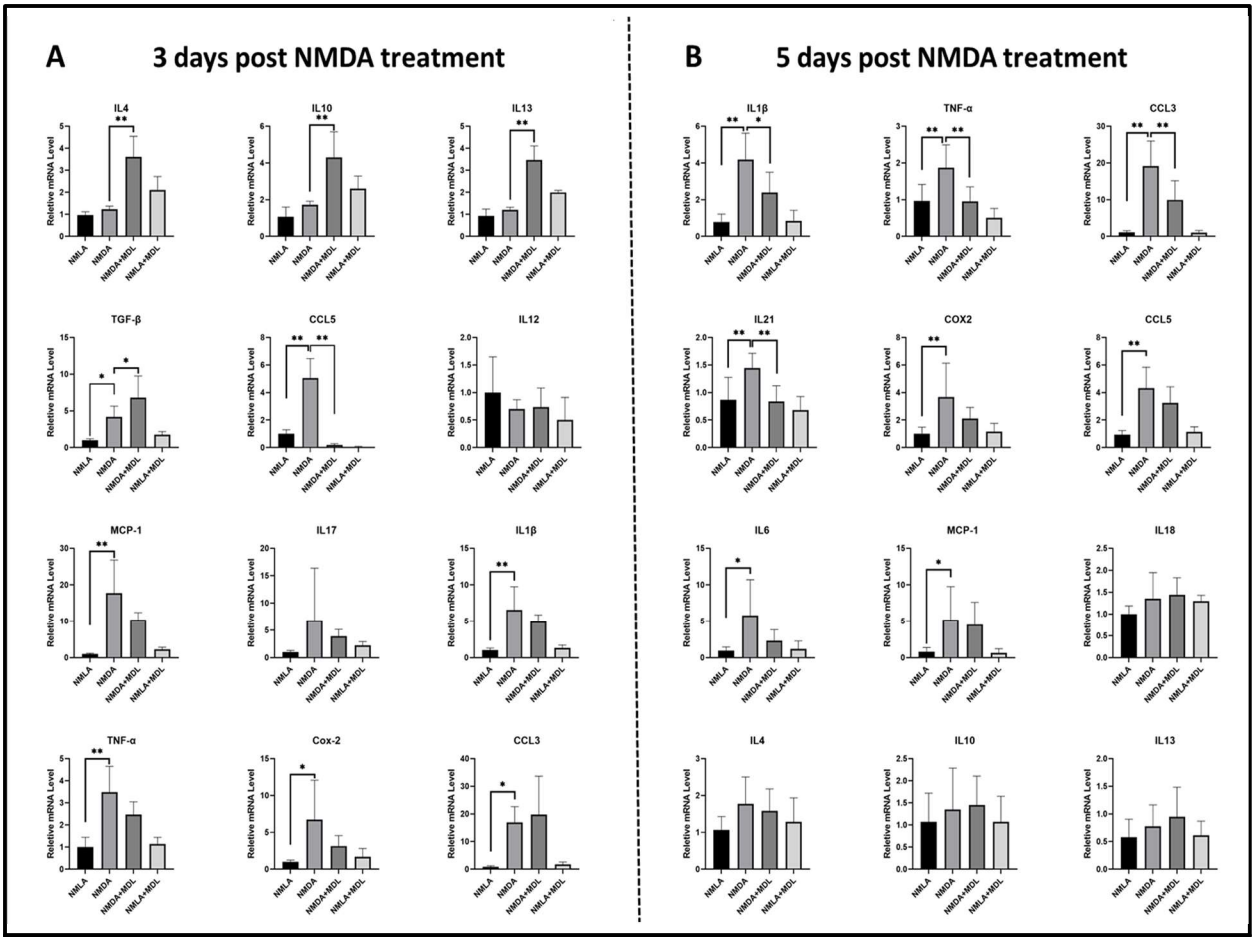

Figure 2.3. Effect of SMOX blockade on cytokines levels in the excitotoxic retinas. Quantitative RT-PCR analysis demonstrating changes in mRNA levels of pro- and anti-inflammatory cytokines and chemokines in excitotoxic retinas following 3 and 5 days post-injury. A) Following 3-day post-injury, in comparison with vehicle treated group, MDL 72527 treated excitotoxic retinas showed significant upregulation in the mRNA levels of anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF-β) and reduced level in the pro-inflammatory CCL5. B) Following 5 days post-injury, the upregulated levels of IL-1β, TNFα, CCL3 and IL-21 mRNA levels were significantly reduced by MDL 72527 treatment in the NMDA groups. The upregulation observed in COX2, and CCL5, are not significantly reduced by SMOX inhibition. IL-6 and MCP-1 were also significantly upregulated in NMDA retinas. The mRNA levels of IL-18, IL-4, IL-10 and IL-13 did not indicate significant changes in any groups. Data are presented as mean ± SD. **p< 0.01; *p< 0.05. N = 5-8 per group.

3.4 MDL 72527 treatment activated antioxidant signaling in the excitotoxic retinas

In the present study, we further investigated if the inhibition of SMOX has an impact on antioxidant signaling pathway. As shown in Figure 2.4, immunoblotting experiments indicated that the blockade of SMOX with MDL 72527 significantly increased the expression of Nrf2 (nuclear factor erythroid 2-related factor 2) in the NMDA retinas compared to vehicle-treated

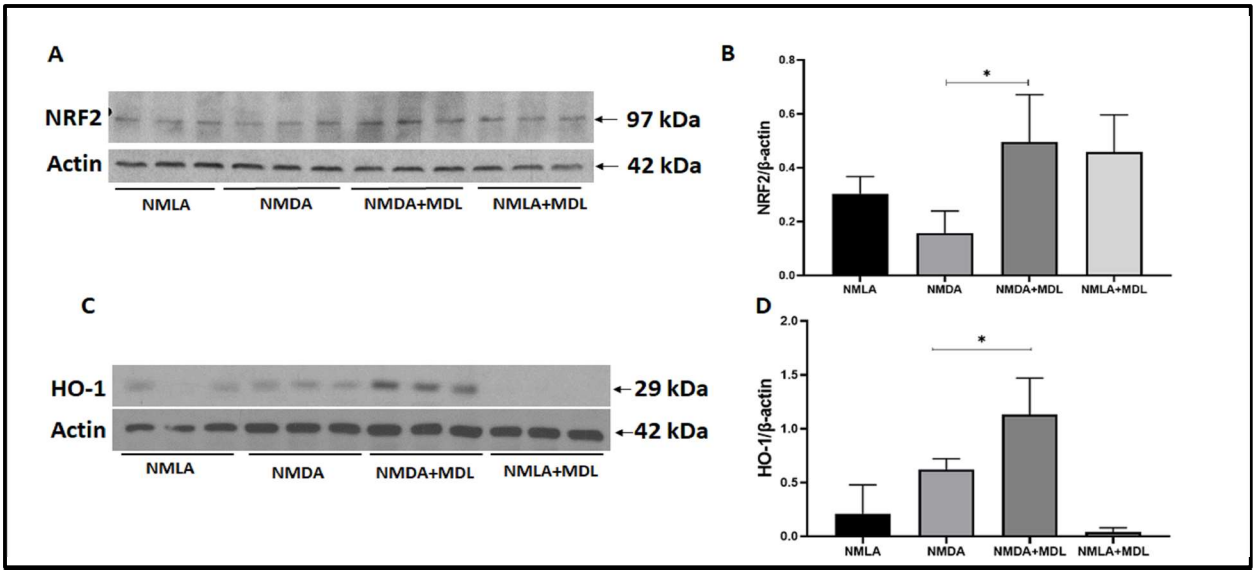

Figure 2.4. Blockade of SMOX by treatment with MDL 72527 increased antioxidant signaling. A&B) Western blot studies and quantification data showing significant increase in Nrf2 expression in MDL 72527 treated excitotoxic retinas in comparison with vehicle treated NMDA group. C&D) Results show that MDL 72527 treatment significantly increased the expression of HO-1 expression in excitotoxic retinas in comparison with vehicle treated group. *P<0.05, N=5-6 per group and representative blots are presented. Results presented as Mean ± SD.

group (Figure 2.4 A&B). Further studies on the expression of heme oxygenase-1 (HO-1), a target gene for Nrf2, indicated that the inhibition of SMOX using MDL 72527 significantly upregulated the expression of HO-1 in the NMDA retinas in comparison to the vehicle-treated NMDA retinas (Figure 2.4 C&D). The changes observed in Nrf2 or HO-1 in NMDA retinas compared to the respective vehicle controls were not significant. These results suggest the potential of MDL 72527 in activating the antioxidant signaling in response to retinal injury.

3.5 Changes in acrolein conjugated proteins in the excitotoxic retinas

Acrolein, a highly reactive aldehyde, directly results from SMOX activation [227] and can elevate oxidative damage by conjugating with proteins, lipids, and DNA [272,273]. In the present study, we studied the levels of protein-conjugated acrolein in NMDA retinas and the impact of SMOX inhibition on their distribution (Figure 2.5A). A significant increase in the level of protein conjugated acrolein was observed in NMDA retinas when compared to the NMLA control group,

and the treatment with MDL 72527 reduced acrolein conjugated proteins to some extent (Figure 2.5A). MDL 72527 markedly reduced acrolein conjugated proteins at a molecular weight around 50-60 kDa (Figure 2.5B). The downregulation observed in the total conjugated proteins and at molecular weight ranges around 25 kDa, however, was not significant in response to MDL 72527 treatment.

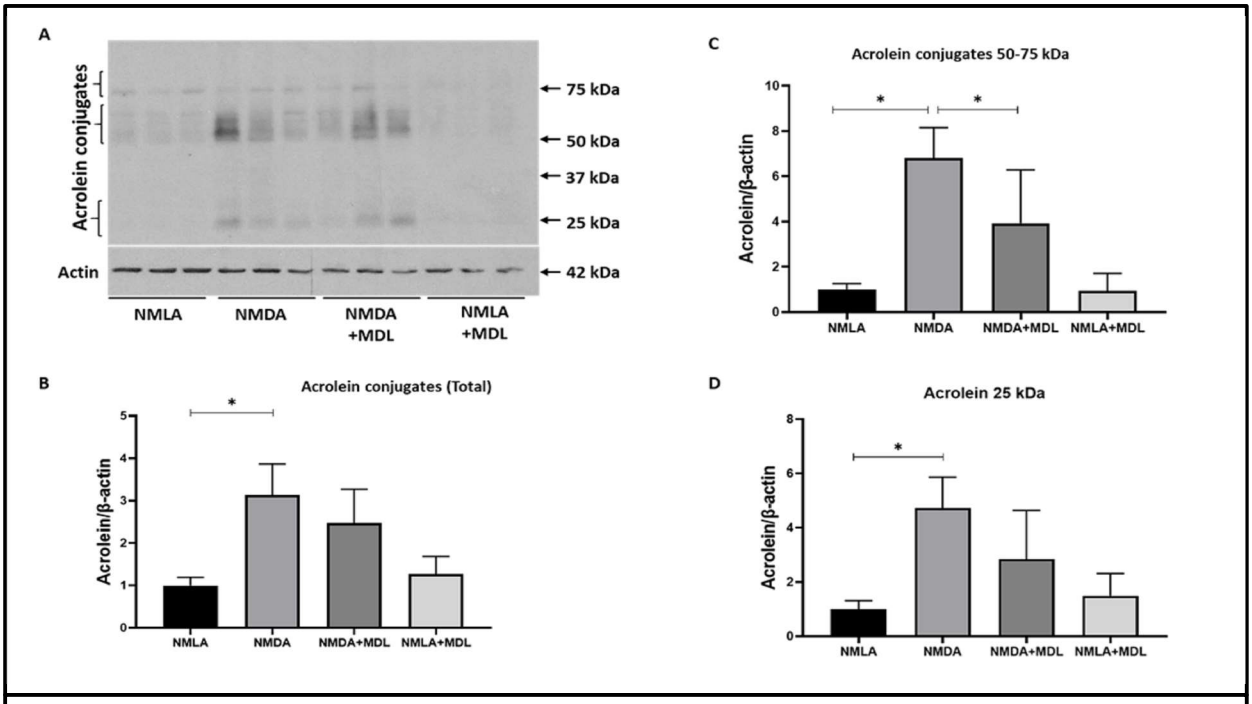

Figure 2.5. Impact of SMOX inhibition on acrolein conjugated proteins. A) Western blot analysis showing the expression of acrolein-conjugated proteins in NMDA treated retinas. B-D) Quantification data showing the marked upregulation of acrolein conjugated proteins at various molecular weight ranges and the effect of MDL 72527 treatment. *P<0.05, N=5-6 per group and representative blots are presented. Results presented as Mean ± SD.

3.6 Impact of acrolein treatment on microglia cells in-vitro

Acrolein is a mediator for oxidative stress [1] and microglia are the primary source for ROS formation in the central nervous system [140]. Using C8-B4 cells with microglial properties, in the current study, we investigated the effect of bovine serum albumin (BSA)-conjugated acrolein on the morphological changes and the formation of reactive oxygen species on microglia. As shown

in Figure 2.6, treatment of C8-B4 cells with 25 µg/ml of BSA-conjugated acrolein for 6 h altered the cellular morphology. Immunostaining using markers CD 11b, F4/80, CD 68, and Iba-1 showed changes such as increase in size and thick processes (Figure 2.6 A-D) in the treated group. H2DCFDA assay showed elevated ROS formation in BSA-acrolein treated cells compared to the control group (Figure 2.6 E&F). Quantification of fluorescence intensity showed significantly upregulated ROS levels in response to acrolein treatment (Figure 2.6 G). These results suggest that SMOX activation could lead to microglial activation and ROS formation.

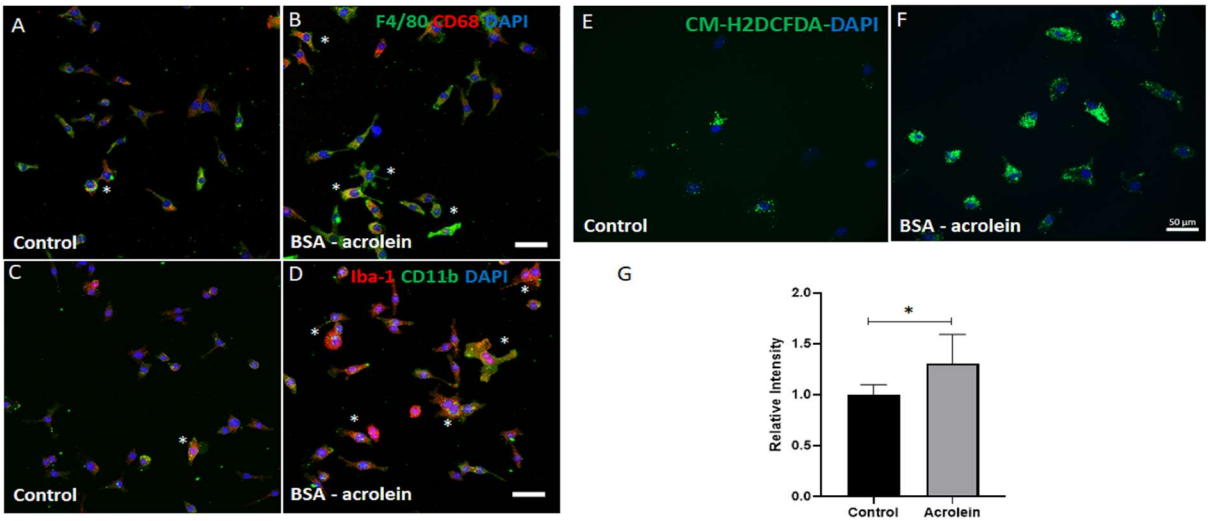

Figure 2.6. Impact of BSA-conjugated acrolein treatment on microglia cells in-vitro. A-D) Confocal images for C8-B4 cells immunostained using F4/80, CD68, Iba-1, and CD11b antibodies showing changes in morphology in response to BSA-acrolein treatment (6h treatment with 25 µg/ml BSA-acrolein). E&F) Images showing the increased ROS levels in C8-B4 cells, studied by H2DCFDA assay and the quantification of ROS levels analyzed by Image J (G). Experiments were repeated minimum 3 times and representative images are presented. *P<0.05. Scale bar=50 µm. Results presented as Mean ± SD.

4. Discussion

The current study provides the first specific evidence for the impact of inhibiting SMOX pharmacologically, using MDL 72527, in limiting neuroinflammation in a mouse model of retinal

excitotoxicity. Retinal excitotoxicity is recognized as a mechanism of neurodegeneration associated with ocular diseases including DR [274]. Previous studies from our laboratory have shown that the SMOX expression is upregulated in the retinal neurons in models of neurovascular injuries and the increase in expression was accompanied by a reduction in neuronal survival, retinal function, and changes in retinal structure [48,237,238]. Inhibition of SMOX with MDL 72527 showed protective effects against these changes [237,238]. However, the molecular mechanisms underlying the neuroprotective effects of SMOX inhibition are not completely understood. Neuroinflammation is a major cause of neuronal damage, and in the present study, we are demonstrating the effect of MDL 72527 on ameliorating inflammation and oxidative stress in a mouse model of retinal excitotoxicity. To the best of our knowledge, this is the first report investigating the impact of SMOX inhibition in excitotoxicity-induced neuroinflammation in the retina.

SMOX is primarily expressed in neurons in the brain [210,211,275] and retina [48,236,238]. A previous study has shown the localization of SMOX in glial cells of fibrovascular tissue from PDR patients [246]. However, the overactivation of SMOX has its effects on other cell types. The reactive aldehydes and H_2O_2 generated as byproducts of SMOX activation are capable of impacting other cells such as glia and endothelial cells [233,237,276-280]. In the recent study by Fan et al using the rat model of cerebral Ischemia/Reperfusion, the authors demonstrated that neuron-derived SMOX induction after stroke was essential for microglial activation and inflammation [211]. Previously, Cervelli et al. showed that neuronal overexpression of SMOX induced microglial activation in the aged neocortex [210]. Targeting SMOX by treatment with MDL 72527 demonstrated neuroprotective effects in rats with ischemic brain damage [207] and traumatic brain injury [208]. A previous study has shown that downregulation of SMOX decreased infarction

volume, neurological deficit, and reduced neuronal apoptosis and inflammation [211]. Studies from our laboratory have indicated that treatment with MDL 72527 also protected retinal neurons in models of oxygen-induced retinopathy (OIR) [236] and diabetic retinopathy [48]. Another study utilizing SMOX-deficient mice showed a reduction in H. pylori-induced inflammation [212]. Similar effects were also noticed when treated with MDL 72527 [213]. Enterotoxigenic Bacteroides fragilis-induced chronic inflammation in C57BL/6 mice was also shown to be reduced by MDL 72527 treatment [281]. However, not many studies have investigated the impact of SMOX inhibition on inflammation associated with retinal neuronal damage and dysfunction.

Studies have shown that NMDA treatment results in increased loss of RGCs and intraocular NMDA injection is useful to study RGCs degeneration in retinal diseases [282-286]. NMDA receptor is a type of glutamate receptors in the mammalian central nervous system [287]. In the retina, NMDA receptors are expressed in both neurons and Müller glia [286,288]. While NMDA receptor activation causes neuronal damage in the retina [289], Müller cells are reported to undergo proliferation [290,291]. Hypertrophy of Müller glia is also a characteristic feature observed in response to excitotoxicity [238,292]. In the diabetic retina, the level of glutamate is increased due to Müller cell dysfunction which is responsible for glutamate metabolism in the retina [293]. Glutamate level is also reported to be elevated in vitreous of glaucoma and diabetic patients [94,294,295]. Accumulation of glutamate and other excitatory molecules activates NMDA receptors and increases calcium influx and causes neuronal cell death due to excessive stimulation [296]. The most affected cells are in the inner retinal layers including ganglion cells, amacrine cells, and bipolar cells [238,297]. Intravitreal injection NMDA induced macrophages/microglia infiltration and increased chemoattractant protein-1 (MCP-1) production in addition to an increase of IL-1β expression [298]. Consistent with these results, our present study demonstrates that the number of

activated microglia/macrophages and pro-inflammatory cytokines are increased in the excitotoxic retinas. However, these changes are reversed in response to MDL 72527 treatment, supporting the impact of SMOX inhibition on reducing inflammation. An earlier study reported from our lab, using the OIR model of neurovascular damage, has also shown suppression of microglia/macrophages with activated morphology and downregulation of inflammatory cytokines, in response to MDL 72527 treatment [237].

Inflammation plays an important role in developing ocular diseases such as DR [109], AMD [250], retinitis pigmentosa [252], and glaucoma [299]. Neuroinflammation is characterized by sustained activation of glial cells such as microglia and astrocyte and the recruitment of other immune cells [300]. Microglia represent the resident immune cells in the retina and it is important to maintain retinal homeostasis; however, they are associated with the progression of diseases such as AMD [301], DR [142], and glaucoma [302]. Microglia in normal retina exhibit ramified morphology but in response to injury microglia exhibit amoeboid morphology and increase the migration and proliferation to the injury's site [303]. In response to various stimuli/insults, the microglia could adopt distinctive phenotypes including the classically activated (M1) state or the alternatively activated (M2) state. The M1-like phenotype is characterized by increased expression of surface markers such as CD16/32, CD68, CD40 and the production of pro-inflammatory mediators including IL-1β, TNF-α, and IL-6 and thus accelerating the inflammatory process [304]. Alternatively, microglia could assume an M2 phenotype, releasing anti-inflammatory, protective and trophic factors, including IL-4, IL-10, 1L-13, and TGF-β and triggering anti-inflammatory responses [305,306]. Activated microglia and macrophages secrete pro-inflammatory mediators (cytokines, chemokines, etc.) and upregulate the expression of inflammatory mediators such as inducible nitric oxide synthase and can act on other cells including neurons [307]. Our findings

show an increase in the number of activated microglia and cells positive for CD 68 and CD 16/32 in response to excitotoxicity, but the treatment with MDL 72527 reversed these effects and upregulated the number of cells in the M2 phenotype. Consistent with these observations, our mRNA results show an early upregulation in anti-inflammatory cytokines followed by a downregulation in pro-inflammatory molecules. RGCs are severely affected in retinal excitotoxicity models. It is an interesting observation in our study that most of the CD 206 positive cells are localized at the GCL of the MDL-treated excitotoxic retina, suggesting the possible role of MDL 72527 in the recruitment of M2 macrophages for RGC survival. Studies have also shown that signals from activated microglia can send signals to Müller cells and cause changes in Müller cells' morphology and function [274]. Reactive Müller cells are also capable of producing pro-inflammatory factors which in turn can increase microglial activation and migration. These bidirectional feedback signals between these two cell types are meant to restore retinal homeostasis in case of injury or disease [154]. In the previous study, we have shown that SMOX inhibition reduced Müller glial injury, as studied by changes in GFAP expression [238].

Acrolein is a toxic aldehyde produced during the oxidation of polyamines [228]. Acrolein can bind with proteins causing their dysfunction [308]. In our study, a significant increase in acrolein conjugated proteins was observed and this was reduced to some extent by MDL 72527 treatment. Further studies are required to identify the specific modifications caused by acrolein. Acrolein has been shown to activate microglia in the CNS [309,310], induce proinflammatory signals, and increase cell migration of microglial cells [229]. However, in addition to acrolein formation, SMOX activation can lead to the formation of H_2O_2 and thus further elevating oxidative stress [205]. In our previous study, we have shown the activation of primary microglia, in response to H_2O_2, another byproduct of SMOX activity [237]. Besides its role in producing inflammatory cytokines, microglia

is also considered a major producer of ROS in CNS [140]. Consistent with these, our study showed acrolein altered morphology and elevated ROS formation in C8-B4 cells. Furthermore, an earlier study showed that treatment of microglial cells treated with supernatant from SMOX-downregulated neurons following OGD/R showed less activation and reduced IL-6 and TNF-α [211].

Oxidative stress and inflammation are closely related pathophysiological processes. Agents with antioxidant and anti-inflammatory properties would be an attractive therapeutic strategy. In the present study, we investigated if the inhibition of SMOX has an impact on antioxidant signals. The stimulation of NMDA receptors by administration of NMDA affects the antioxidants status [311]. The nuclear factor erythroid 2-related factor 2 (Nrf2) plays an important role in regulating several antioxidant enzymes [312], and its activation protects the retina from retinal diseases [313]. Nrf2 expression protected against oxidative stress in retinal diseases such as DR [314] and AMD [315]. In our study, we found that the treatment with MDL 72527 increased the expression of Nrf2 and its downstream target HO-1 regulated by Nrf2. HO-1 is an inducible protein in response to different stimuli and its expression is reported to be increased as a defense in the retina in cases such as diabetic retinopathy [316], ischemia-reperfusion [317], and light-induced damage [318]. Induction of HO-1 expression showed a protective effect on RGCs in diabetic rats by reducing inflammation, apoptosis, and proliferation effects in the diabetic retina [316]. Induction of HO-1 expression also reduced the retinal damage against IR injury [319]. Taken together, MDL 72527 treatment reduced the production of acrolein and reduced the activation of the microglial cell.

In summary, the inhibition of SMOX by the treatment with MDL 72527 reduced microglia/macrophage activation, induced changes in polarization status, downregulated proinflammatory cytokines release, decreased acrolein formation, and activated the antioxidant

signals in the model of retinal excitotoxicity. A proposed mechanism of SMOX mediated neuroinflammation and neurodegeneration in the retina is depicted in Figure 2.7. Our studies report for the first time the specific effect of SMOX inhibition, using MDL 72527 treatment, in limiting neuroinflammation in the retina. We have demonstrated a crucial role for the SMOX signaling pathway as one of the major mechanisms associated with inflammation and neurodegeneration in the excitotoxic retina. Considering the need for new therapies for patients suffering from ocular diseases associated with retinal neuroinflammation such as diabetic retinopathy or glaucoma, our results are highly relevant from a clinical perspective. Our findings suggest that targeting SMOX signaling can be considered as a therapeutic strategy to treat neurodegenerative diseases of the eye.

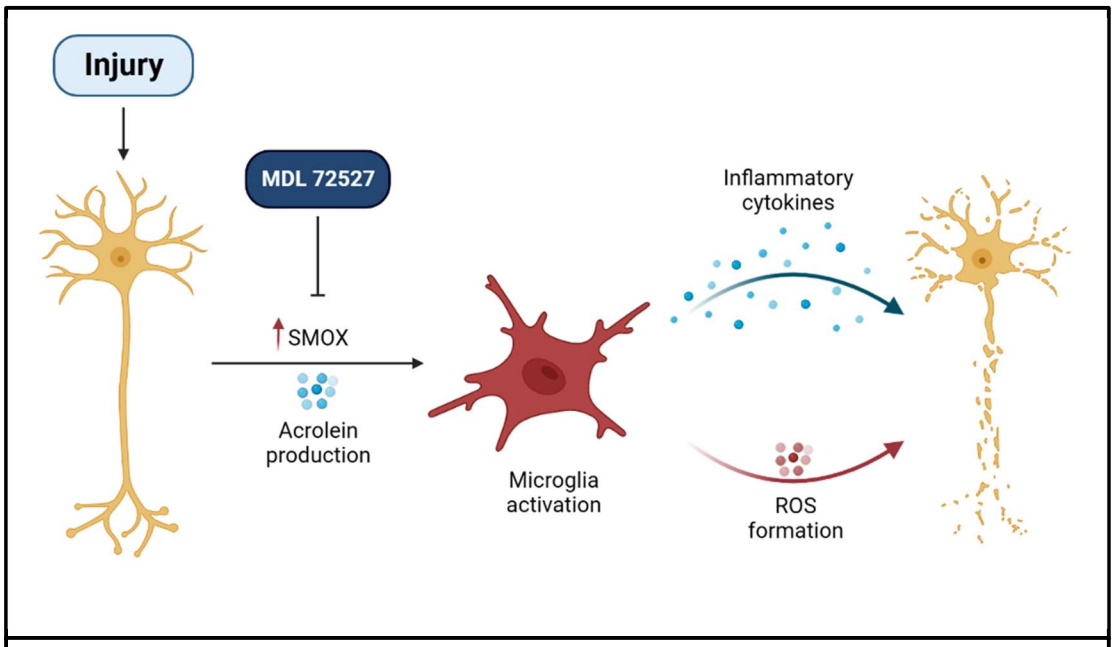

Figure 2.7. Proposed mechanism of excitotoxicity-induced neuronal damage. A diagrammatic representation of the proposed molecular mechanism of SMOX mediated neuroinflammation and oxidative stress and neurodegeneration in the retina. Created in BioRender.com

CHAPTER III: SPECIFIC AIM 2

Long-term Treatment with MDL 72527 Improved Visual Acuity, Contrast Sensitivity, Inner Retinal Function and Reduced Retinal Neuro-glial Injury in Diabetic Mice[2]

[2] Moaddey Alfarhan, Fang Liu, Payaningal R. Somanath, and S. Priya Narayanan.
To be sumbitted to *Diabetologia*

Abstract

Diabetic retinopathy, a major cause of vision loss, is characterized by neurodegenerative changes in the retina. A previous study from our laboratory demonstrated that 12-week treatment with MDL 72527, a pharmacological inhibitor of spermine oxidase (SMOX), reduced neuronal damage and improved retinal function in diabetic mice. The current study was undertaken to assess the effectiveness of long-term SMOX inhibition in visual acuity, contrast sensitivity, inner retinal function, and neuroglial injury in diabetic mice. Diabetes was induced in male mice (C57BL6J, 8-10 weeks old) with repeated intraperitoneal streptozotocin injections (65 mg/kg/ citrate buffer, up to five times). The control mice received citrate buffer injections. Mice with blood glucose higher than 350 mg/dL were considered diabetic and maintained 24 weeks post-diabetes. Animals (diabetic or controls) were treated with MDL 72527 (20 mg/kg) or saline three times a week. While bodyweight and blood glucose were measured weekly, glycated hemoglobin was tested every eight weeks. Mice were evaluated for visual acuity (VA) and contrast sensitivity (CS) (optokinetic tracking response, every eight weeks), and retinal function (electroretinography, 24 weeks). Retinal cryostat sections and fresh frozen retinas were used for immunofluorescence and for western blot studies of neuronal and glial markers. Diabetic mice showed a significant reduction in measures of VA and CS following 8, 16, and 24 weeks of diabetes onset. However, treatment with MDL 72527 improved both VA and CS in diabetic mice at the time points studied with a significant increase at 24 weeks post-diabetes compared to non-diabetic mice. The significant decrease observed in inner retinal function (scotopic b wave amplitudes) in diabetic mice compared to non-diabetic controls was improved by MDL 72527 treatment. Diabetes-induced reductions in synaptophysin, neuronal-specific class III tubulin (Tuj-1) and glutamine synthetase (GS) and an increase in vimentin were reversed in response to SMOX inhibition. Results observed in MDL 72527 treated control mice were similar to that of the saline-treated group. In conclusion,

our findings show that SMOX inhibition reduced diabetes-induced neuroglial damage and improved visual acuity and inner retinal function in diabetic mice. Targeting SMOX signaling may provide a new strategy for reducing vision loss in diabetes.

Keywords: diabetic retinopathy, neurodegeneration, SMOX, MDL 72527, visual acuity, contrast sensitivity, electroretinography

1. Introduction

According to International Diabetes Federation (IDF) Diabetes Atlas, people affected by diabetes was estimated to be 537 million globally, while this number is expected to reach 643 million in 2030 and 2045 to 783 million [320]. The estimated number of patients with diabetic retinopathy (DR) in 2020 was 103 million globally, expected to reach 160 million by 2045 [8]. It is anticipated that patients with vision-threatening DR are expected to reach around 50 million in 2045 [8]. Studies have shown that the prevalence of DR is higher in type 1 diabetic (T1D) patients than in patients with type 2 diabetes (T2D) [321]. Also, patients with T1D showed a higher prevalence of proliferative diabetic retinopathy (PDR) than T2D patients [321]. While DR is recognized as a neurovascular disease, the existing treatments target vascular complications at the late stages of the disease and exhibit unfavorable side effects [175].

Neurodegeneration is a hallmark of diabetic retinopathy [33,34]. Several reports have shown that retinal neurons become dysfunctional and undergo degeneration during the initial stages of diabetes [36,322-326]. Postmortem samples from diabetic patients showed many apoptotic cells in the retinal layers, and the number of apoptotic cells was higher in the ganglion cell layer (GCL) [327]. Studies on experimental models also showed an increase in apoptotic cells in diabetic retinas, particularly within the GCL [36,322]. In addition, the retina of diabetic patients showed changes in electroretinography (ERG), which occurred at the early stages of the disease [323-326]. Diabetic patients also showed a reduction in visual function and changes in retinal structure at the early stage of the disease [25,27,50,51,328]. However, the specific mechanisms by which neurons in the diabetic retina undergo degeneration are incompletely understood. Further studies on the

molecular mechanisms underlying diabetes-induced retinal neuronal damage and dysfunction will assist in identifying new therapeutic targets for DR.

Dysregulation of polyamines and polyamine metabolism have been noticed in different neurological diseases, such as Alzheimer's disease [329], Parkinson's disease [209], and traumatic brain injury [330]. Studies from our laboratory have shown that the oxidation of polyamines is associated with neurovascular damage in the retina [48,270,331,332]. We have demonstrated that spermine oxidase (SMOX, an enzyme in the polyamine catabolism) is critically associated with neuronal damage and dysfunction in retinal disease models [48,270,331,332]. SMOX expression is upregulated in the retinas of diabetic patients and diabetic mice models [48,246]. A previous study from our laboratory demonstrated that upregulation of SMOX in the retina of streptozotocin (STZ)-diabetic mice (12 weeks post-induction) was associated with neurodegenerative changes such as retinal thinning and altered retinal function [48]. However, treatment with MDL 72527 (N1, N4-bis (2,3-butadienyl)-1,4-butane diamine), a pharmacological inhibitor of SMOX [234], improved these changes [48]. In addition, MDL 72527 treatment demonstrated neuroprotective properties in multiple disease models [48,331-334]. The long-term effects of SMOX blockade on experimental models of diabetes and its impact on vision loss are not known yet. In the present study, using MDL 72527 treatment, we investigated the effectiveness of long-term SMOX inhibition in visual acuity, contrast sensitivity, inner retinal function, and neuro-glial injury in diabetic mice.

2. Methods

2.1 Animals

In this study, we used 8-10 weeks-old male mice (C57BL6J) (Jackson Laboratories, Bar Harbor, ME, USA). All experimental procedures were performed in accordance to ARVO Statement for the Use of the Animals in Ophthalmic and Vision Research. All procedures conducted were approved by the Institutional Animal Care and Use Committee of Augusta University, Augusta, GA, USA and the Charlie Norwood VA Medical Center, Augusta, GA, USA. All efforts were taken to minimize the pain and animal suffering during the experimental procedures.

2.2 Induction of diabetes

In this study, the experimental model of type 1 diabetes was induced using streptozotocin (STZ) treatment. STZ-induced diabetes is a well-established model to study the complications of diabetic retinopathy [335]. STZ destroys β-cells of the pancreatic islets which results in insulin deficiency, hyperglycemia, polydipsia, and polyuria [336]. These symptoms mimic type 1 diabetes in humans [336]. Several laboratories are using this model for studying diabetes-induced neurovascular changes in the retina. Only male mice were used because previous studies have shown that female mice are resistant to STZ [337]. Diabetes was induced with repeated intraperitoneal STZ injections (65 mg/kg/ citrate buffer, up to five times). The control mice received citrate buffer injections. Animals with 350 mg/dl glucose and over were considered diabetic and maintained for 24 weeks post-diabetes onset (Fig 3.1A).

2.3 MDL 72527 administration

Mice were divided into four groups, as vehicle-treated controls (Con+Veh), vehicle-treated diabetic (Db+Veh), MDL 72527-treated diabetic (Db+MDL), and MDL 72527-treated control

(Con+MDL) as depicted in (Fig 3.1B). Mice in Con+MDL and Db+MDL groups received 20 mg/kg of MDL 72527 in saline intraperitoneally (IP) every three days immediately after the onset of diabetes and continued for 24 weeks. Mice in Con+Veh and Db+Veh groups received saline injections. The dose of 20 mg/kg every three days was used based on our previous studies, which showed protective effects against the diabetic retina with no adverse effects [48]. Furthermore, an

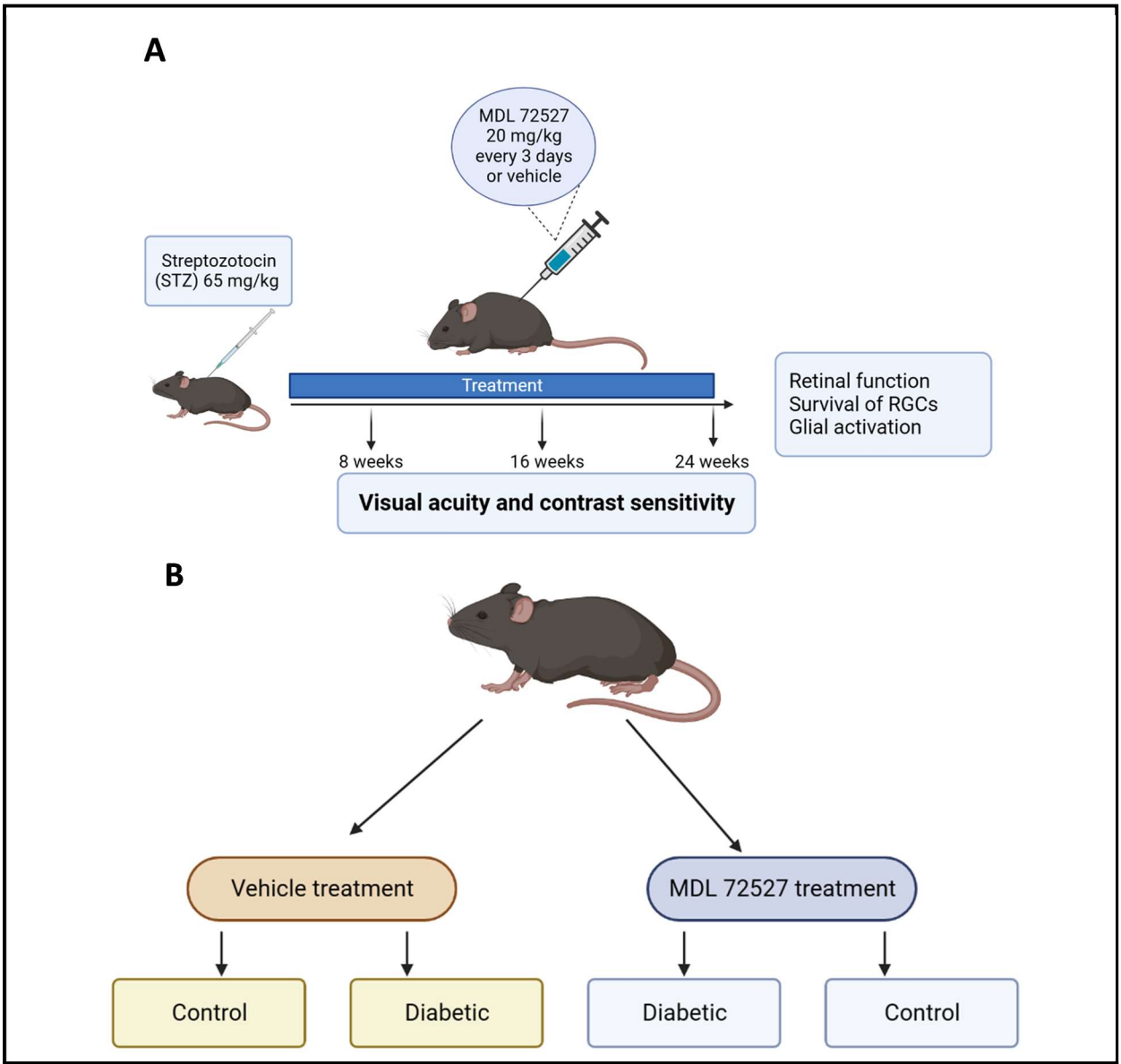

Figure 3.1. Experimental design. Mice (8-10 weeks, C57/BL6J male) were used in the study. (A) Mice in the diabetic group received five injections of 65 mg/kg of streptozotocin (STZ). Animals were treated with either MDL 72527 (20 mg/kg, every 3 days) or saline. (B) Mice were divided into four groups according to the type of treatment. Created in BioRender.com

intraperitoneal injection was used as the route of administration because some studies that used MDL 72527 by adding to drinking water might cause toxic effects by the accumulation of spermine in the blood, which has been reported after long-term exposure to MDL 72527 [234,338].

2.4 Body weight, blood glucose, and HbA1c

Body weight and blood glucose were measured weekly. Blood glucose levels were determined using the Alpha TRAK2 blood glucose monitoring system. In addition, hemoglobin A1c was assessed every eight weeks using Mouse Hemoglobin A1c (HbA1c) Assay Kit (catalog# 80310, Crystal Chem, USA).

2.5 Optokinetic tracking response

Mice were subjected to a virtual reality chamber to evaluate visual behavior (OKT; Opto-Motry, Cerebral Mechanics, Alberta, Canada) available at the Augusta University vision core, according to the published methods [339,340]. These experiments were performed following 8, 16, and 24 weeks of diabetes. Briefly, the mouse was placed in the center of the arena, surrounded by four monitors. Vertical black and white waves were projected on the screens and moved at a speed of 12 d/second. 100% contrast was used to measure spatial frequency (SF) thresholds. A staircase procedure was used in which the observer evaluated low to high visual acuity. Contrast sensitivity was measured on SF 0.092; while the contrast started at 100% contrast, it systematically reduced until the threshold was identified.

2.6 Electroretinography

ERGs were performed after 24 weeks of diabetes using Touch/Touch feature of the Celeris Ophthalmic Electrophysiology System (Diagnosys, Lowell, MA) available at the Augusta

University vision core, according to the published methods [339,340]. The Touch/Touch protocol stimulates one eye at a time and uses the fellow, unstimulated eye used as the reference. For scotopic ERGs, mice were dark-adapted for approximately 16 h prior experiment, then tested using a series of light flashes of increasing energy (0.001, 0.005, 0.01, 0.1, 0.5, and 1.0 cd s/ m^2).

2.7 Immunoblotting

Protein analysis using the Western blot method was performed according to the standardized methods in our laboratory [48,270,331,332]. Samples were homogenized in 1X RIPA lysis buffer (Millipore, Billerica, MA, USA) containing protease inhibitor cocktail (Thermo Scientific™) and phosphatase inhibitor cocktail (Thermo Scientific™). Protein concentration was estimated using BCA Protein Assay Kit (Thermo Scientific™). Approximately 7.5-15 µg of protein samples were separated on 10–12% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane and blocked in 5% non-fat dry milk in 1X Tris-buffered saline with 0.1% Tween (TBST). Membranes were incubated with respective primary antibodies (Table 3.1). Next, membranes were washed with TBST and then incubated with respective secondary antibodies (anti-rabbit or anti-mouse HRP-conjugated secondary antibody) for one hour at room temperature. Protein bands were detected using enhanced chemiluminescence (ECL) (Thermo Fisher) and the ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using ImageJ software and normalized to the loading control.

2.8 Immunostaining

Immunostaining of retinal cryostat sections was performed per our laboratory's established methods [48,270,331,332]. Briefly, eyes were removed and fixed in 4% paraformaldehyde overnight at four degrees and cryoprotected in 30% sucrose for 24 hours. Cryostat sections (10 µm) obtained

on glass slides were permeabilized and blocked in 10% normal donkey serum with 0.1% Triton X-100 for one hour. Next, sections were incubated with primary antibodies (Table 3.1). Then, sections were washed three times with PBS and then incubated with fluorescein-conjugated secondary antibodies for 1-2 hours at room temperature. Next, the sections were washed in PBS and covered with a mounting medium with DAPI (Vectashield; Vector Laboratories, Burlingame, CA). Images were obtained using a Leica STELLARIS confocal microscope.

Antibody	Catalog Number	Source	Dilution	Experiment
Actin	4970S	Cell Signaling Technology	1:1000	Western blot
GAPDH	5174S	Cell Signaling Technology	1:1000	Western blot
Glutamine synthetase	GTX630654	GeneTex	1:200	Immunostaining
Glutamine synthetase	GTX630654	GeneTex	1:10000	Western blot
NeuN	MAB377	EMD Millipore	1:200	Immunostaining
Synaptophysin	MABN1193	EMD Millipore	1:200	Immunostaining
Synaptophysin	MABN1193	EMD Millipore	1:25000	Western blot
Tuj-1 (Neuron-specific beta-III Tubulin)	MAB1195	R&D Systems	1:200	Immunostaining
Tuj-1 (Neuron-specific beta-III Tubulin)	MAB1195	R&D Systems	1:4000	Western blot
Vimentin	M072529-2	Agilent Technologies	1:1000	Western blot

Table 3.1. Antibodies used in the study.

2.9 Statistical analysis

GraphPad Prism 9 was used for statistical analysis. Statistical analysis was performed using one-way ANOVA followed by the Tukey test for multiple comparisons. Results were considered significant when $P < 0.05$.

3. Results

3.1 Body weight, blood glucose, and HbA1c measurements

Blood glucose and body weight were measured weekly. Blood glucose levels remained elevated in diabetic mice during the study period (Fig. 3.2A). There is a marked decrease in body weight in the diabetic mice compared to the control group as the duration of diabetes increases (Fig. 3.2B). HbA1c was measured every eight weeks, and all the mice in diabetic groups showed elevated HbA1c level (Fig. 3.2C). The measurements on the completion of 24 weeks showed that mice in the diabetic groups have significantly higher blood glucose and HbA1c levels than the non-diabetic control groups (Table 3.2). The final body weight measurements for both groups of diabetic mice were significantly lower than the mice in the control group. MDL 72527 treatment did not show any effect on the blood glucose levels, body weight, or HbA1c in diabetic mice (Table 3.2).

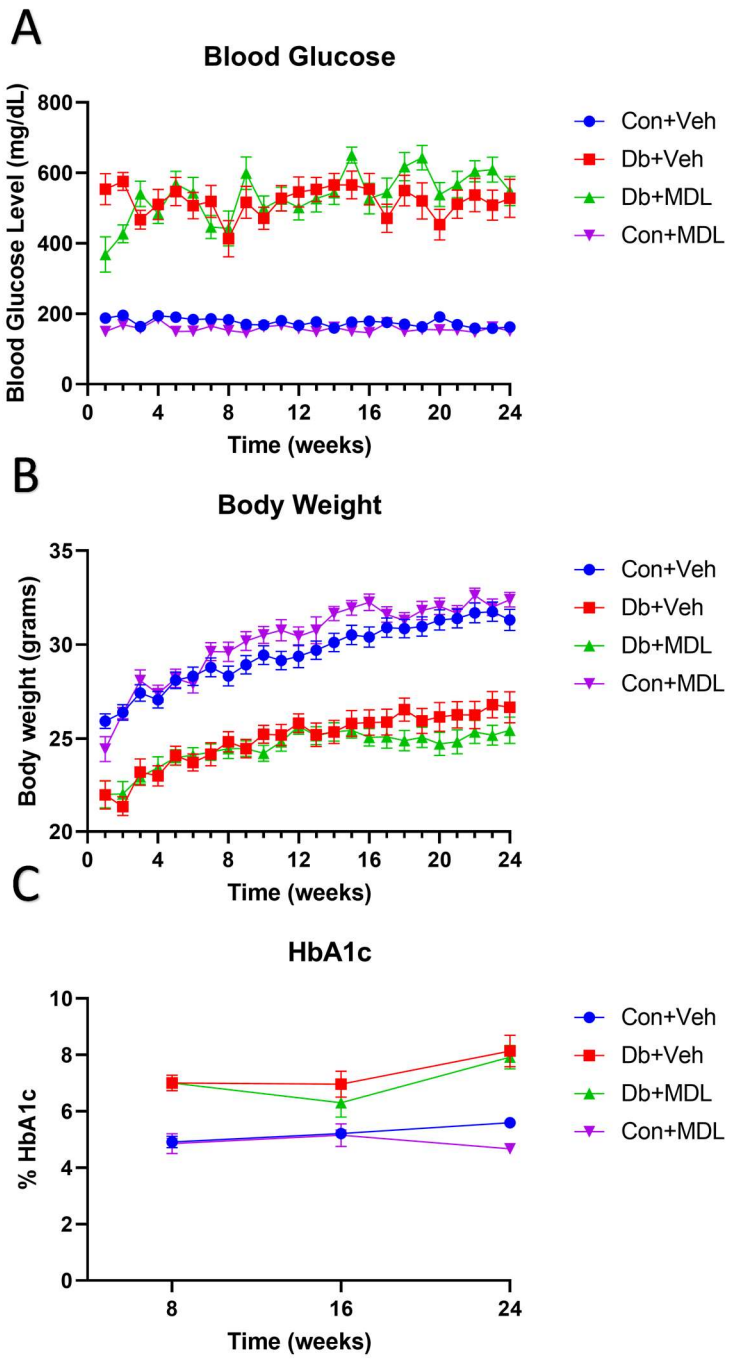

Figure 3.2. Changes in blood glucose, body weight, and HbA1c during the treatment period. Changes in blood glucose (A) and body weight (B) were measured weekly, and that of HbA1c was evaluated every 8 weeks (C). Data presented as mean ± SEM. N=10-16 per group.

Group	Body weight (g)	Blood Glucose (*mg/dL*)	HbA1c (%)
Control + Vehicle	31.32 ± 2.2	163 ± 11	5.6 ± 0.76
Diabetic + Vehicle	26.67 ± 3.2*	527 ± 210*	8.1 ± 2.2*
Diabetic + MDL	25.45 ± 2.5*	549 ± 150*	7.9 ± 1.5*
Control + MDL	32.39 ± 1.23	151 ± 11	4.7 ± 0.5

Table 3.2. Changes in body weight, blood glucose, and HbA1c following 6 months of diabetes. Data represented as mean ± SD. In compared to control + vehicle, both diabetic groups have shown a significant reduction in body weight and a significant increase in blood glucose level and the level of glycated hemoglobin (HbA1c). (*P<0.01, N=10-15 per group).

3.2 MDL 72527 treatment improved visual acuity and contrast sensitivity in diabetic mice

Visual acuity and contrast sensitivity were assessed following 8, 16, and 24 weeks of diabetes onset at a speed of 12°/s. Mice at eight weeks of post-diabetes showed a significant reduction in visual acuity and contrast sensitivity (P<0.01) compared to the control group (Fig. 3.3A and B). MDL 72527 treatment showed improvements in visual acuity and contrast sensitivity. However, these changes were not statistically significant. Visual acuity and contrast sensitivity were further reduced considerably in vehicle-treated diabetic mice following 16 weeks of diabetes compared to vehicle-treated control mice (P<0.01)(Fig. 3.3C and D). The changes observed in response to the treatment with MDL 72527 were not significant. Upon the completion of 24 weeks of treatment, the mice in vehicle-treated diabetic group showed a significant reduction in visual acuity and contrast sensitivity (P<0.01)(Fig. 3.3E and F). Significantly improved visual acuity and contrast sensitivity were observed in diabetic mice that received the treatment with MDL 72527 in (P<0.05)

when compared to vehicle-treated diabetic mice (Fig. 3.3E and F). No noticeable changes were

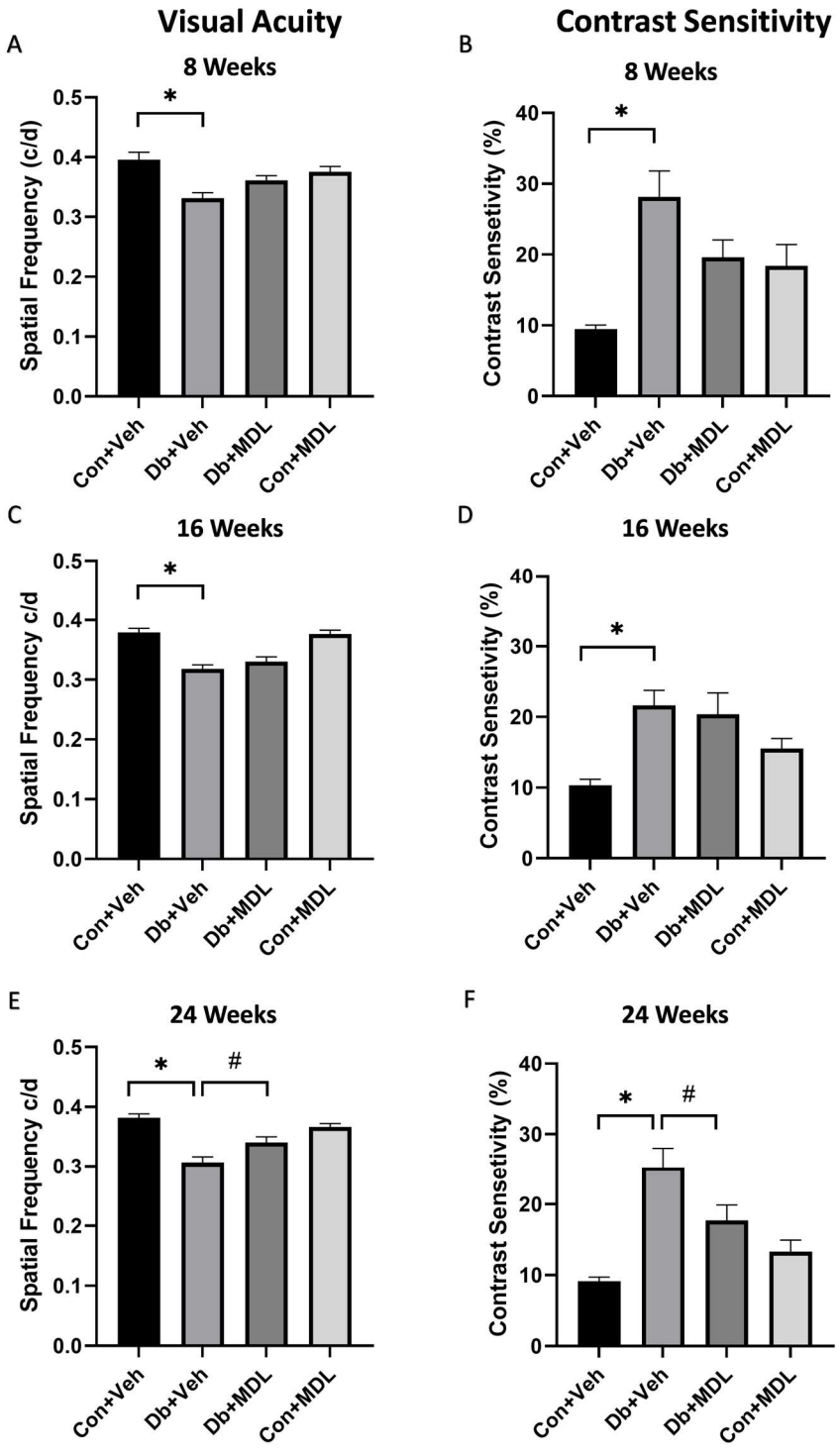

Figure 3.3. Changes in visual acuity and contrast sensitivity. Optokinetic measurements showing changes in the visual acuity and contrast sensitivity in control and treatment groups at 8 weeks (A and B), 16 weeks (C and D), and following 24 weeks of diabetes (E and F). Data are shown as mean ± SEM. N=10-17 per group. *P< 0.01, #P < 0.05.

observed in the control mice that received MDL 72527 treatment.

3.3 Effect of SMOX inhibition on ERG responses

ERG studies were performed 24 weeks following diabetes induction. Notable changes were not observed in the scotopic a-wave responses across the four groups at low light intensities (0.001, 0.005, and 0.01 cd/s/m^2) (Fig 3.4A). On higher flash intensities (0.1, 0.5, and 1 cd/s/m^2), diabetic mice treated with the vehicle showed significantly lower amplitude than the non-diabetic control mice ($P<0.01$; Fig 3.4A). However, treatment with MDL 72527 in diabetic mice exhibited an improvement in scotopic a-wave amplitude when compared to vehicle diabetic mice ($P<0.01$; Fig 3.4A). Diabetic mice treated with vehicle showed significant reductions in scotopic b wave amplitude in all studied flash intensities (0.001, 0.005, 0.01, 0.1, 0.5, and 1 cd/s/m^2) when compared to the control group ($P<0.01$; Fig 3.4B). However, MDL 72527 treatment of the diabetic mice exhibited a significant improvement in scotopic b wave amplitudes compared to vehicle-treated diabetic mice, at all intensities studied (Fig 3.4B). These data indicate that treatment with MDL 72527 improved rods' function, which counts for around 97% [341] of photoreceptor cells and improved inner retinal function. Responses from mice in the control group received MDL 72527 treatment were similar to that of vehicle-treated controls.

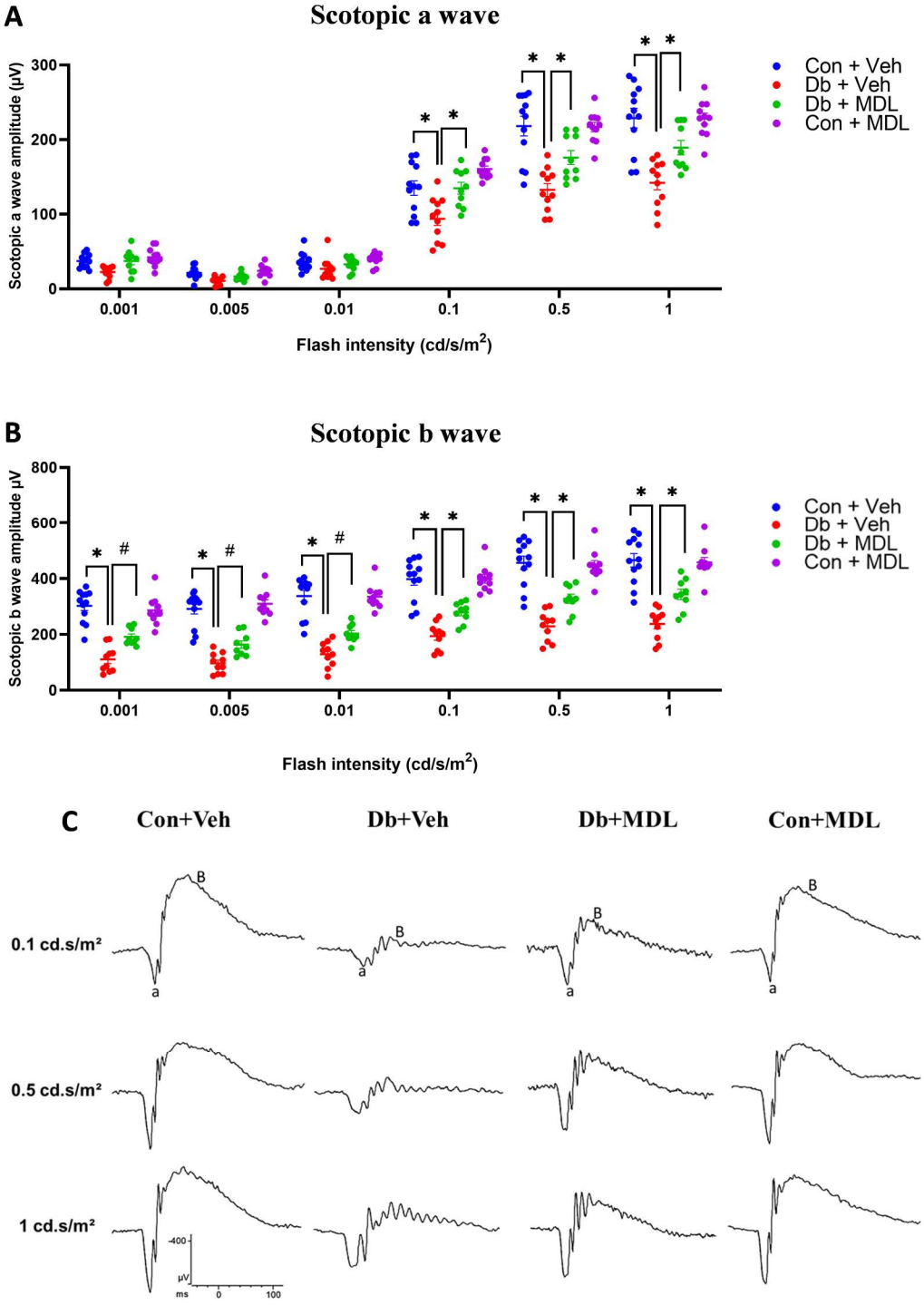

Figure 3.4. Effect of MDL 72527 treatment on diabetes-induced functional changes in the retina. Electroretinography studies demonstrating changes in scotopic ERG measurements performed following 24 weeks of diabetes. Changes in scotopic a wave (A) and b wave (B) amplitudes studied at flash intensities ranging from 0.001 to 1.0 cd/s/m^2. (C) Representative scotopic ERG responses at 3 light intensities 0.1, 0.5, and 1 cd.s/m^2. Scotopic or dark-adapted electroretinogram. Data are shown as mean ± SEM. N=9-12 per group. *P< 0.01; #P<0.05.

3.4 Effect of SMOX inhibition on neuronal survival

We investigated the changes in neurons in the diabetic retina. Tuj-1 is a neuronal marker expressed in retinal ganglion cells (RGCs) and their axons [342-344]. In this study, we employed both immunoblotting and immunofluorescence staining experiments to assess the changes in Tuj-1 expression in all four groups following 24 weeks of diabetes. Results from Western blot studies showed that Tuj-1 is significantly reduced in the diabetic retina ($P<0.01$) (Fig. 3.5A and B). While the treatment with MDL 72527 upregulated Tuj-1 expression in the diabetic retinas, the changes were not statistically significant (Fig. 3.5A and B). MDL 72527 treatment did not show a difference between the two control groups. Immunostaining results show reduced Tuj-1 levels in

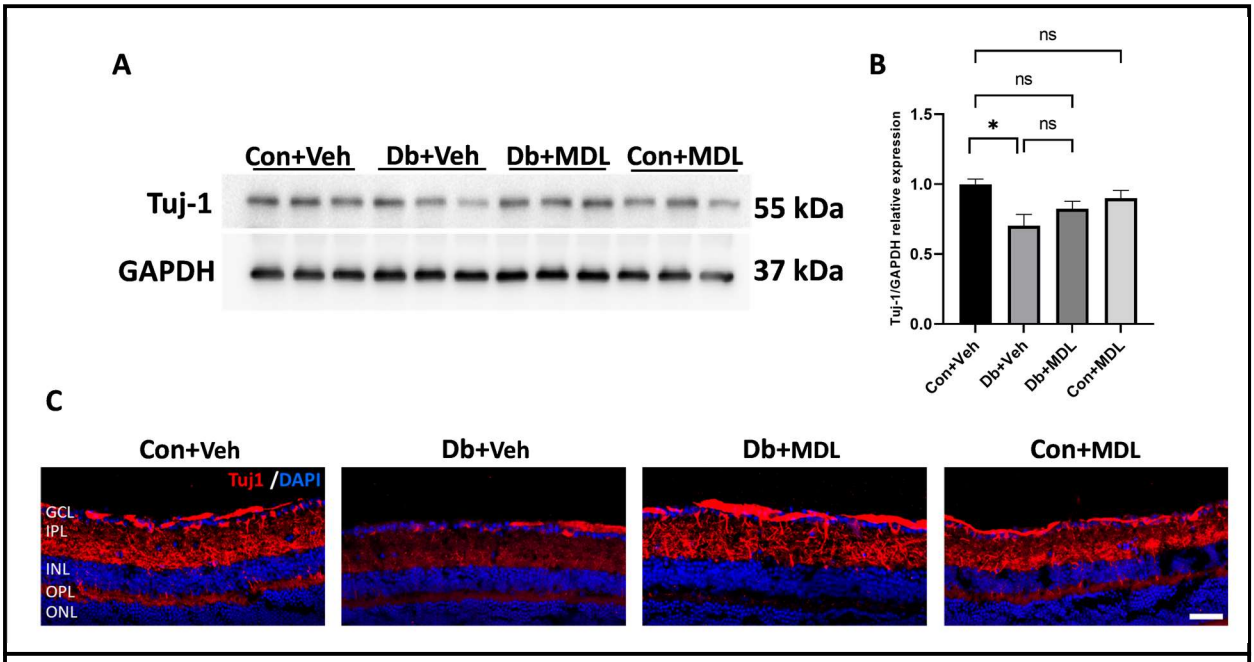

Figure 3.5. Effect of MDL 72527 treatment on the expression of Tuj-1 after 24 weeks of diabetes. (A and B) Immunoblotting studies show changes in the expression of Tuj-1 in diabetic retinas and the effect of MDL 72527 treatment. Quantification of intensity measurement demonstrates a significant reduction in diabetic retinas, however, the upregulation in response to MDL 72527 is not significant. Data are presented as mean ± SEM. (N=5-6; *P<0.01) (C) Confocal images show the expression of Tuj-1 in the retinal sections of diabetic mice and the impact of MDL 72527 treatment. Scale bar 50 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; Tuj-1 (Neuron-specific beta-III Tubulin).

the RGCs and their axons in the diabetic retina, which was improved in response to SMOX inhibition (Fig. 3.5C).

Synaptophysin is a presynaptic protein associated with synaptic vesicles [345]. Synaptophysin, expressed in the inner and outer plexiform layers, is essential in forming and recycling synaptic vesicles [346-350]. Twenty-four weeks post-diabetes, diabetic mice who received vehicle showed a significant reduction in the expression of synaptophysin when compared to the non-diabetic control ($P<0.05$) (Fig. 3.6A and B). Diabetic mice treated with MDL 72527 showed a slight upregulation in the synaptophysin level. However, this was not statistically significant (Fig. 3.6A and B). No significant difference in synaptophysin expression was observed between the control groups. Confocal images obtained from retinal cryostat sections immunostained with synaptophysin showed reduced expression in the diabetic retina, which was improved by MDL 72527 treatment (Fig. 3.6C).

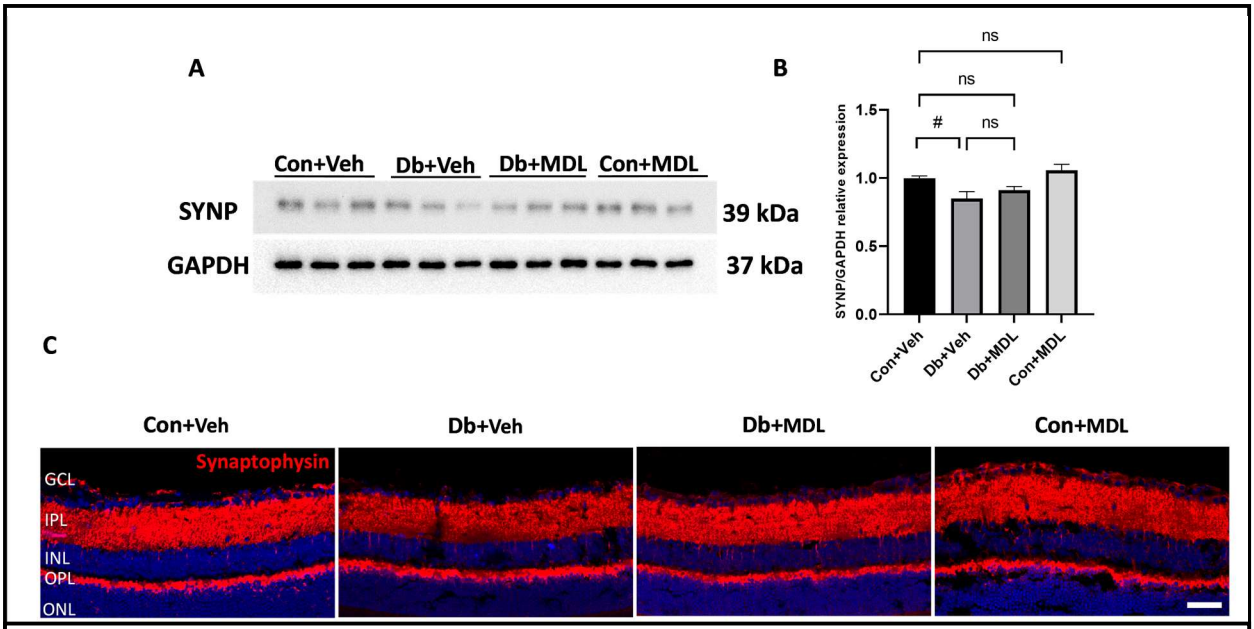

Figure 3.6. Effect of MDL 72527 on Synaptophysin expression. (A and B) Western blot studies showing changes in the expression of synaptophysin in the retinas of diabetic mice, and the impact of MDL 72527. Data are presented as mean ± SEM. (N=6; #$P<0.05$) (C) Representative confocal images showing the changes in the expression of synaptophysin in the retinal sections. Scale bar 50 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; SYNP, synaptophysin.

Neuronal-specific nuclear protein (NeuN) is generally considered a marker of mature neurons [345] and is being used as a marker for RGCs. Confocal images show the loss of RGCs in the retina of untreated diabetic mice (Fig. 3.7A). Quantitative analysis of NeuN-positive cells on the retinal sections s showed a reduction in the number of NeuN-positive cells in the retinas of diabetic mice. Treatment with MDL 72527 increased the NeuN-positive cells in the diabetic retina. However, these changes were not statistically significant (Fig 3.7 B).

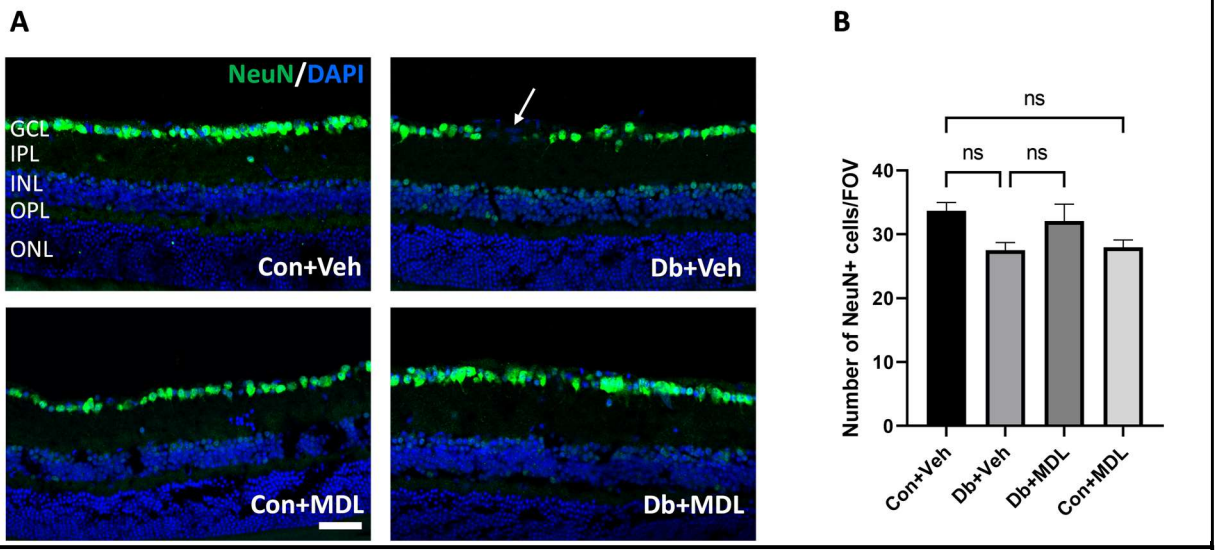

Figure 3.7. Effect of long-term diabetes on RGCs survival. (A) Representative confocal images of NeuN immunostaining, showing the loss of RGCs in diabetic retinas. White arrow indicates area of RGCs loss. (B) Quantitative analysis shows the changes in the number of NeuN+ positive cells per field of view. Data are presented as mean ± SEM (N=4). Scale bar 50 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

3.5 Effect of SMOX inhibition on retinal glial damage

Glial injury is a characteristic feature of diabetic retina [117]. Glutamine synthetase (GS) and vimentin are markers of Müller cells [351]. GS is expressed in Müller cells, which converts neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) to glutamine [352]. In this study, immunoblotting studies indicated a significant downregulation of GS protein in the diabetic retinas from vehicle-treated mice, compared to that of non-diabetic controls ($P<0.05$), which was reversed in the diabetic retinas from the

MDL 72527 treated group (P<0.01) (Fig 3.8 A and B). Representative images of immunostained retinal sections represent the downregulation of GS in the diabetic retina, which was reversed in response to SMOX inhibition (Fig. 3.8C). No marked changes were evident in the control retinas.

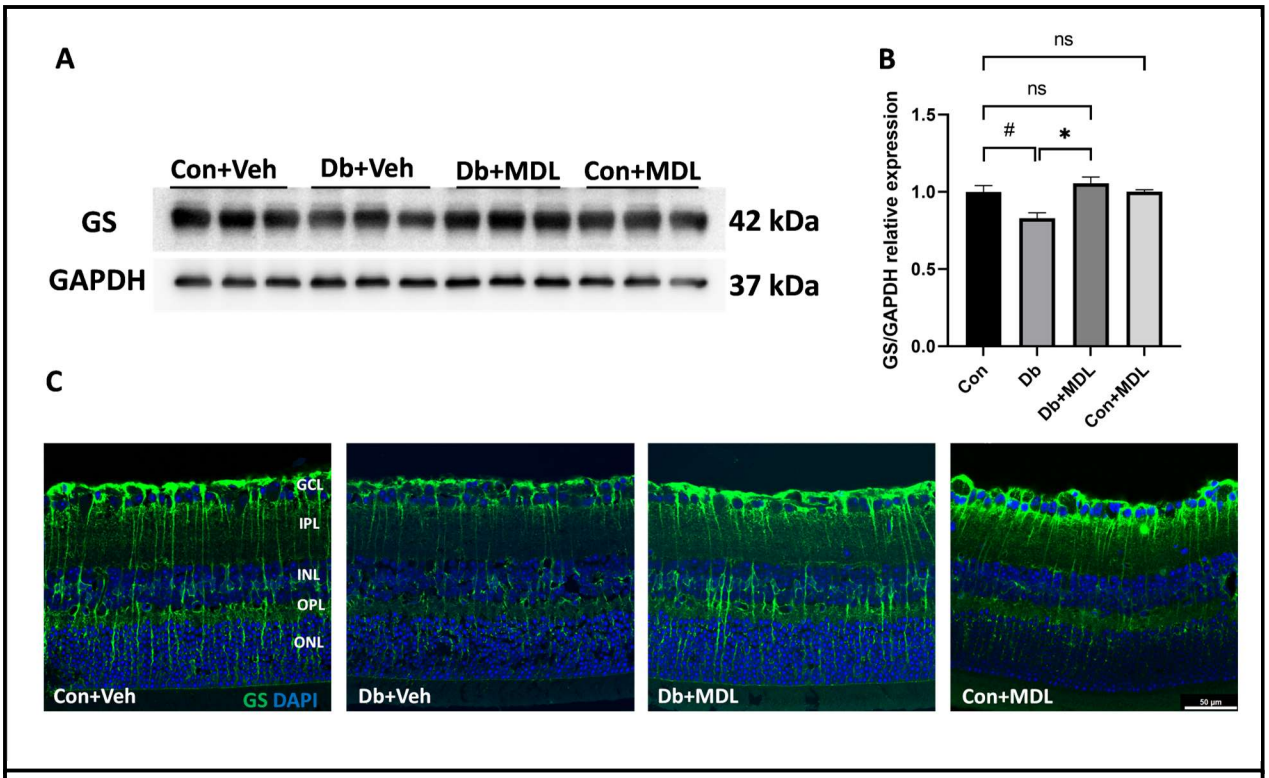

Figure 3.8. Effect of MDL 72527 treatment on the expression of glutamine synthetase. (A and B) Western blot data showing the changes in the expression of GS in the retinas from different groups. Data are presented as mean ± SEM (*P< 0.01, #P < 0.05 N=3). (C) Representative confocal images for glutamine synthetase showing its expression in retinal layers. Scale bar 50 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

Vimentin is expressed in both astrocytes and Müller cells [353]. The expression of vimentin increases with retinal injury [353]. Our results showed that vimentin expression significantly increased in the retinas of diabetic mice in comparison to the mice in vehicle-treated control group (Fig. 3.9). Interestingly, treatment with MDL 72527 significantly decreased the diabetes-induced upregulation vimentin expression in the retina. (Fig. 3.9 A and B). There was no significant difference between non-diabetic groups.

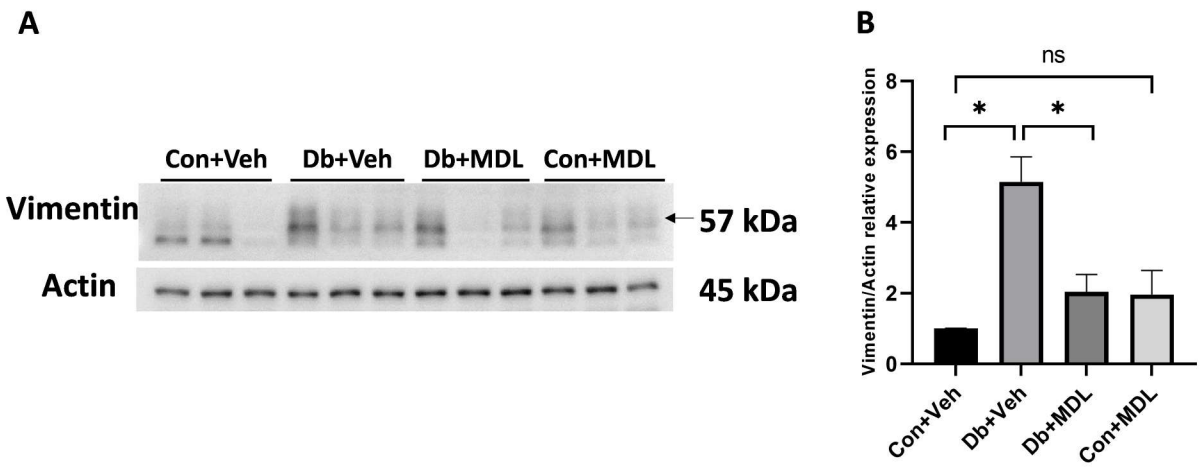

Figure 3.9. Effect of MDL 72527 treatment on vimentin expression in diabetic retinas. (A&B) Western blot experiments showing changes in the expression of vimentin in diabetic retinas, and the impact of MDL 72527 treatment. Data are shown as mean ± SEM, N=5-6. *P<0.01.

4. Discussion

Neurodegeneration in the diabetic retina is characterized by loss of visual acuity [25,27,50,328], alterations in ERG [323-326], and loss of RGCs [327]. Current treatments for DR are for the late stage of the disease and mainly target vascular injury. Intravitreal injections of anti-vascular endothelial growth factor (VEGF) are being used to treat PDR [354]. However, the inhibition of VEGF in diabetic retinopathy experimental models increased neuronal apoptosis, significantly reduced the number of RGCs and amacrine cells and worsened the visual acuity [171,172]. Interestingly, VEGF treatment increases neuronal survival in the ex-vivo model of diabetic retinopathy [171]. On the other hand, several neuroprotective treatments that protect retinal neuronal cells also protect against changes in retinal vasculature [35]. Therefore, it is essential to find a better therapeutic approach targeting neuronal changes in diabetic retinopathy.

SMOX expression increased in plasma in patients with stroke [355]. The mRNA expression and protein expression of SMOX are elevated in brain penumbra in rats after ischemic brain injury [207,211]. Inhibition of the activity of SMOX in rats after ischemic brain injury reduced the

ischemic infarcted volume, reduced neurological deficit, and reduced inflammation [207,211]. Pharmacological inhibition of SMOX using MDL 72527 reduced the water content and necrotic cavitation after traumatic brain injury [208]. By contrast, SMOX overexpression in mice neocortex led to increase oxidative stress and gliosis and reduced neuronal cells in response to excitotoxic injury [210]. Studies from our lab have shown that SMOX expression increased in the retina in models of ischemic retinopathy and excitotoxicity [331,332]. Furthermore, we have reported that inhibition SMOX using MDL 72527, a selective inhibitor for SMOX activity [356], protected against retinal neuronal death, retinal inflammation, and upregulated antioxidant signaling in various ocular disease models [48,270,331-334]. Utilizing the STZ-induced model of diabetes, we investigated the impact of long-term inhibition of SMOX on retinal neuronal damage and dysfunction. Our results indicate that MDL 72527 treatment improved visual acuity, contrast sensitivity, retinal function and reduced neuro-glial damage in the diabetic mice. To the best of our knowledge, this is the first report investigating the long-term effect of SMOX inhibition on diabetes–induced neurodegeneration, suggesting the potential benefits of SMOX inhibition to treat diabetic retinopathy.

Clinical studies have shown that visual acuity and contrast sensitivity are reduced in diabetic patients at an early stage of the disease, even before vascular changes [25-27,357]. Visual acuity in diabetic mice showed a significant reduction after four weeks of diabetes and continued until up to 10 months post-diabetes [41,172]. Similarly, contrast sensitivity is also reduced in diabetic animals [41,172,358,359]. Consistent with previous studies, our results indicated reductions in visual acuity and contrast sensitivity in diabetic mice. Another major characteristic of DR is the alterations in retinal function, indicated by changes in ERG responses reported in DR patients [323-326] and experimental models [47-49], and our results further confirm the alterations in ERG

responses. The protection offered by MDL 72527 in improving visual acuity, contrast sensitivity, and retinal function in diabetic mice indicates its beneficial effects. Tuj-1 is a reliable marker for RGCs and axonal integrity [344,360]. In vitro experiments using Tuj-1 as a neuronal marker, high glucose treatment inhibit neuronal stem cell differentiation to neuronal cells [361]. The expression of Tuj-1 was also reduced in neuronal cells in response to high glucose and hydrogen peroxide (H_2O_2) exposure [361]. In line with experimental studies that showed a reduction in diabetic retinas [48,360], our results show that long-term diabetes led to a significant decrease in the expression of Tuj-1 in diabetic mice. Synaptophysin, a transmembrane protein found in presynaptic neurotransmitter vesicles, involves release and recycling neurotransmitters [362,363]. Synaptophysin is reduced in the hippocampus of female diabetic mice, and the improvement in synaptophysin is linked to protection against cognition impairment [364]. Expression of synaptophysin in retinas was reduced in diabetic animals, and that reduction was associated with a decline in retinal function [365,366]. Our results also have shown that diabetic mice showed a significant decrease in the expression of synaptophysin. The upregulation offered by MDL 72527 in the levels of Tuj-1 and synaptophysin in the diabetic retina further supports that SMOX inhibition reduced retinal neuronal damage resulting from long-term diabetes.

Loss of retinal ganglion cells is a hallmark of diabetic retinopathy. STZ-diabetic mice showed a significant reduction in the number of RGCs at the early, mid, and late stages of the disease [172]. NeuN is a neuronal marker used in retinal studies as a marker for RGCs [367-369]. A previous study using the STZ-induced diabetic rat model has shown that NeuN-positive cells at GCL were significantly reduced in the early stages of diabetes (during 1 to 4 months of diabetes induction); however, the loss of NeuN-positive cells at GCL was not significant during the later stages (after 6 and 12 months) of diabetes [370]. Another study reported that STZ-diabetic mice

showed a remarkably higher number of apoptotic cells at the GCL within the two weeks of diabetes, but these numbers were not significant between 1 to 6 months of diabetes [371]. Studies from our laboratory are in line with these observations. While we observed a considerable reduction in RGCs in mice following 16 weeks of diabetes compared to their non-diabetic controls [48], the changes were not statistically significant following 6 months of diabetes. These could possibly resulting from various factors such as the changes in the markers used for RGCs, survival of different subpopulations of RGCs during the disease progression, or related age loss in RGCs.

Injury to glial cells is another feature of the diabetic retina [117]. GS expressed in Müller cells converts glutamate to glutamine which is less active metabolite [372]. GS in Müller cells protects against risk of increase in glutamate level and retinal excitotoxicity [90,372,373]. Retinas of diabetic animals showed a reduction in mRNA and protein expression of GS, and that reduction increased with the duration of diabetes [374]. Furthermore, this reduction in GS was associated with deterioration in visual function [374]. Our study results agree with reduced GS levels in the diabetic retina. Vimentin is a marker for glial cell activation expressed in Müller cells and astrocytes [375]. Experimental models of diabetes demonstrated significant increase in vimentin expression following 2 to 6 months of diabetes [172,375]. These observations support our findings on the high expression of vimentin in diabetic retina. Vimentin plays a role in photoreceptors degeneration in mice following retinal detachment [353].

SMOX activation is considered as one of the main source of acrolein formation endogenously [214]. Acrolein is a reactive aldehyde that has a toxic effect on different cell types [224]. On the cellular level, acrolein can cause DNA and protein adduction, increase oxidative stress, disruption of mitochondrial function and cellular membranes [224]. In neurodegenerative diseases, acrolein formation and accumulation play a role in disease as spinal cord injury

[222,225,377], brain infarction [221,378], Alzheimer's disease [219], and multiple sclerosis [220,379]. Studies from our lab have shown an increase in conjugated acrolein in GCL and inner nuclear layer (INL). This increase was associated with retinal neuronal damage in experimental models of diabetic retinopathy and multiple sclerosis [48,333]. The treatment MDL 72527 in these two models reduced the formation of acrolein conjugates and reduced retinal neuronal damage in models of diabetes and multiple sclerosis [48,333]. A previous study has shown that Müller cells under in vitro hypoxic conditions produced H_2O_2 and acrolein, which were reduced by treatment with MDL 72527 [246]. Other studies have shown that acrolein exposure to Müller cells increased reactive oxygen species (ROS) formation and glial migration [380,381]. Exposure of microglia to acrolein has also increased cell migration and cyclooxygenase 2 (COX-II) and ROS production [229,334]. In vivo study using STZ-treated diabetic rats demonstrated that treatment with acrolein scavenger for three months reduced glial cell activation and improved retinal function [107]. We have reported previously that inhibition of SMOX using MDL 72527 also reduced glial activation in a model of retinal excitotoxicity [331]. These data suggest that lowering acrolein conjugates protects against retinal glial cell activation. In the present study, MDL 72527 treatment reduced vimentin levels and upregulated the expression of GS in the diabetic retina. Altogether, these observations, along with the improvements in ERG responses, suggest that long-term inhibition of SMOX protects against retinal neuro-glial damage in diabetic mice.

In conclusion, the present study indicated that pharmacological inhibition of SMOX improved visual acuity, contrast sensitivity, retinal function, and reduced neuro-glial injury in an experimental model of type 1 diabetes. Hence, targeting SMOX signaling may provide a new strategy for reducing vision loss in diabetes.

CHAPTER IV

Conclusion

Diabetic retinopathy continues to be the major cause of visual impairment and blindness in working-age adults [382]. Studies have shown that neurodegeneration in the diabetic retina occurs at the early stages of the disease and characterized by reduced visual function [25,27,50,328], changes in ERG responses [325,326], and loss of RGCs [327]. Current treatments for DR are for the late stages of the disease when the visual function is already affected. In addition, these treatments could be associated with undesired side effects such as infections, moderate vision loss, or increased ocular pressure [175]. The expected increase in the number of DR patients in the coming years [320] and the unavailability of early intervention emphasize the need for a better and early intervention targeting neuronal changes in diabetic retinopathy that can prevent or slow down the progression of the disease.

Studies from our laboratory have shown that SMOX (a critical enzyme in the polyamine metabolic pathway) expression is increased in models of oxygen-induced retinopathy [332], retinal excitotoxicity [331], diabetic retinopathy [48], and optic neuritis [333]. Furthermore, the increase in SMOX expression was associated with neuronal damage and dysfunction along with elevated oxidative stress, inflammation, and glial activation [48,270,331-333]. MDL 72527 is a selective irreversible inhibitor of SMOX with demonstrated neuroprotective properties [356,383-385]. Studies

from our lab showed that SMOX inhibition using MDL 72527 protected against retinal neuronal death and retinal inflammation in ischemic retinopathy [270,332], retinal excitotoxicity [331], and optic neuritis [333]. Genetic downregulation of SMOX in the brain reduced the activation of microglial cells and inflammatory cytokines, including TNF-α and IL-6, after ischemic brain injury [211]. In the ischemic retina, SMOX inhibition with MDL 72527 reduced the number of activated microglia and reduced the expression of inflammatory cytokines, including IL-1β, IFN-γ, TNF-α, and IL-6 [270]. In addition to its role in inflammation, SMOX activation also increases oxidative damage by increasing H_2O_2 in the ischemic retinas [332]. However, the role of SMOX activation in neuroinflammation and oxidative stress has not been studied in neurodegenerative models, including diabetic retinopathy.

The main purpose of this study was to better understand the role of SMOX on retinal neurodegeneration in diabetic retinopathy, with a specific focus on the molecular mechanisms of neurodegeneration and the impact of SMOX inhibition on vision loss. Employing the NMDA-induced retinal excitotoxicity, a well-established retinal neurodegeneration model [286], studies under specific aim 1 investigated the mechanistic role of SMOX on neuroinflammation and oxidative damage in the retina. Our results demonstrated that treatment with MDL 72527 significantly reduced the number of M1 microglia/macrophages (activated phenotype), a significant decrease in inflammatory cytokine/chemokine levels, including IL-1β, TNF-α, IL-21, CCL3, and CCL5, in the excitotoxic retinas. On the other hand, MDL 72527 treatment protected retinal inflammation by increasing the number of microglia/macrophages of M2 phenotypes using CD206 and arginase-1 markers, associated with increased expression of anti-inflammatory molecules, including IL-4, IL-10, IL-13, and TGF-β. In addition, MDL 72527 treatment upregulated antioxidant signaling, shown by increased protein levels of Nrf-2 and HO-1. In vitro

studies using C8-B4 (microglia/macrophage) cells showed that exposure to conjugated acrolein (a byproduct of SMOX activation) led to morphological changes and a significant increase in ROS formation. These results are consistent with previous studies on ischemic brain injury [211] and ischemic retina [270,332], that the increase in SMOX activity causes neuroinflammation and oxidative damage.

While results from aim 1 and previous studies demonstrated the neuroprotective and anti-inflammatory effects of SMOX inhibition using MDL 72527 on retinal excitotoxicity and diabetic retinopathy models, the long-term impact of SMOX inhibition on the retina remained unknown. Therefore, the studies under the second aim evaluated the long-term effects of SMOX inhibition in diabetic mice. Utilizing the STZ-induced diabetic mouse model, a well-established model mimicking type-1 diabetes in humans [335,386]. The optokinetic parameters were significantly reduced in the diabetic mice at three different time points studied. MDL 72527 treated diabetic group showed improved visual acuity and contrast sensitivity after 8 and 16 weeks of diabetes, with a significant increase after 24 weeks of diabetes. These results were supported by ERG analysis performed following 24 weeks of diabetes, which revealed that diabetes-induced a significant reduction in scotopic ERG responses; however, SMOX inhibition significantly improved the scotopic a and b amplitudes in diabetic mice, indicating improved inner retinal and photoreceptor functions. The decline in both visual functions and ERG amplitudes found in vehicle-treated diabetic mice was also associated with reductions in neuronal marker Tuj-1, which is a marker for RGCs and their axons [342-344] and synaptophysin, a presynaptic protein [387]. However, treatment with MDL 72527 improved the expression of these two markers in the diabetic retina. While GS (the enzyme expressed in Müller cells converts glutamate to less active metabolite) is significantly reduced in the retinas of vehicle-treated diabetic mice, treatment with

MDL 72527 increased the GS levels. Vimentin is a marker for reactive glia that increases in diabetic retinas [375]. Our results further showed that vimentin expression significantly increased in diabetic retinas while it was reduced in response to SMOX inhibition. Overall, the findings of improved visual function, ERG amplitudes, and expression changes in neuronal and glial markers suggest that long-term treatment with MDL 72527 protects against retinal neuronal dysfunction and vision loss caused by diabetes.

In conclusion, our findings showed that pharmacological inhibition of SMOX, using MDL 72527 treatment, protected the retina against retinal inflammation and oxidative injury. Furthermore, SMOX inhibition improved visual acuity and retinal function and reduced neuro-glial damage caused by diabetes. Our findings suggest that SMOX plays a vital role in diabetic-induced neurodegeneration by inducing inflammation and oxidative stress in addition to Müller cell dysfunction (Fig 4.1). Hence targeting SMOX signaling using MDL 72527 may provide a new and early intervention strategy for reducing vision loss in diabetes.

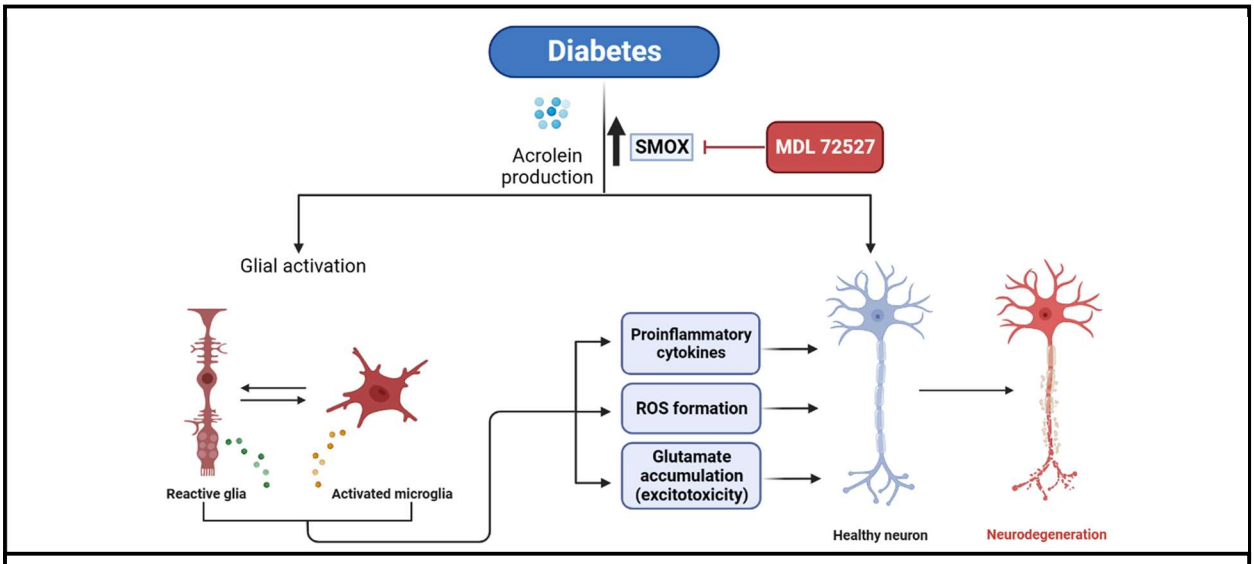

Figure 4.1. Proposed role of SMOX in neurodegeneration in the diabetic retina. SMOX activity increases in diabetic retinas, which results in acrolein formation. Acrolein causes neuronal damage in addition to activation of both glial types (macroglia and microglia). Müller cells become unable to metabolize glutamate, which increases neuronal toxicity. Activated microglia cause additional damage to neurons by releasing inflammatory cytokines and ROS formation. Created in BioRender.com

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