ROLE OF NEURONAL SPERMINE OXIDASE IN VASCULAR DAMAGE IN A MODEL OF ISCHEMIC RETINOPATHY

by

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ABSTRACT

Retinal vascular injury is a major cause of vision impairment in ischemic retinopathies such as diabetic retinopathy and retinopathy of prematurity (ROP). Current treatments for ischemic retinopathies target the late stage of the disease and have long-term side effects. Therefore, there is a great need to identify novel mechanisms and therapeutic approaches. Previous studies from our laboratory have shown the critical role of spermine oxidase (SMOX) in retinal neurovascular damages. Utilizing the oxygen-induced retinopathy (OIR) mouse model of ROP, the current study was undertaken to investigate the impact of neuronal SMOX in mediating retinal vascular injury. Newborn mice (WT control, SMOX Tg, or WT treated with MDL 72527/saline) along with their mother were maintained in 70% oxygen from postnatal day (P) 7 to 12 followed by normoxia until P17. Animals were sacrificed at P17 and eyes were processed for different experiments. Our data demonstrated that retinal vascular development was normal in the SMOX Tg retina, and MDL 72527 treatment had no deleterious effect on WT retinas. Staining of retinal flat-mounts with Isolectin B4 showed that SMOX overexpression significantly increased OIR induced avascular area and pathological neovascular tufts in SMOX Tg retinas compared to WT-OIR retinas. Treatment with SMOX inhibitor, MDL 72527, significantly reduced these pathological changes.

Furthermore, Glial fibrillary acidic protein (GFAP) immunostaining of retinal cryostat sections showed elevated glial activation in SMOX Tg-OIR retinas compared to WT-OIR retinas. Western blot analysis of retinal lysates performed on OIR groups treated with MDL 72527/vehicle demonstrated increased levels of acrolein conjugates compared to normoxia control groups. Considering the urgent need to identify new therapeutic modalities for retinal neovascular diseases, our data suggest that blockade of SMOX signaling can be a potential therapeutic target to limit the progression of ischemic retinopathies.

INDEX WORDS: Ischemic retinopathy; Oxygen induced retinopathy; Spermine Oxidase; Vascular injury; MDL 72527; Neovascularization; Vaso-obliteration; Gliosis

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Pharm.D., Jazan University, Saudi Arabia, 2015

A thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2021

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DEDICATION

I dedicate this thesis to my lovely parents (Layla and Ahmad), for their unwavering support and encouragement, faithful prayers, and unconditional love.

I also dedicate this work to my siblings (Mohammad, Ageela, Mariam, Sultan, and Ibrahim), for their irreplaceable support, guidance, and motivation.

ACKNOWLEDGEMENTS

First and foremost, I am beyond grateful to Allah for helping me to achieve this work.

I would like to thank my supervisor, Dr. Priya Narayanan, for her support, guidance, and patience throughout the last two years. I greatly appreciate everything she did for me. I also thank my advisory committee members, Dr. Duo Zhang and Dr. Somanath Shenoy, for their feedback, support, suggestions, and expertise. I thank the Retinopathy laboratory members, for their time, assistance, and technical support. A special thanks to the Clinical and Experimental Therapeutics program for the incredible training experience that has given me and for the future opportunities has provided.

Finally, I cannot thank my family enough for their unconditional love and endless support. I thank my employer, Jazan University, for the financial support. Far more individuals than I can list here deserve gratitude for supporting me and making this research and training possible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Retinopathy of prematurity (ROP): an overview

ROP is a form of proliferative retinal disease that affects premature infants. Globally, ROP is regarded as the leading cause of childhood blindness [1]. The extent of prematurity is directly correlated with the severity of the disease. In the USA, ROP affects about 14.000 premature infants annually. Of those afflicted, 1.000 to 1.5000 develop severe disease status that requires medical intervention [2] A multicenter study illustrated that the incidence of any form of ROP in infants weighing <1251 g was 68% [3]. Furthermore, about 15 million infants are born prematurely every year (less than 37 weeks of age). This number presents premature infants at a high risk of ROP, especially in less developed and low-income countries, where expert ophthalmologists are less available to early screen and detect this disease [4]. The improvement of neonatal care in the developed countries poses a high risk of ROP among survived premature infants [5]. Taken together, the risk of ROP is omnipresent. So far, the screening of ROP is primarily based on birth weight (BW) and gestational age (GA) while the etiology of ROP is multifactorial. Therefore, a timely screening is mandated upon child birth and continues throughout childhood to detect and treat ROP [6].

The two standing factors that contribute to ROP are birth weight and gestational age. As revealed by Schaffer et al. In infants with BW ≤1251g, the chance of developing ROP decreases by 27%, with each 100 g increase in BW, and each additional week in GA is associated with a reduction in developing ROP by 19% [7]. Another causative factor is oxygen, entailing both the duration and

concentration. The risk of developing ROP is double, with every 12 hours of partial pressure of oxygen (PO2) ≥80mmHg [8]. Other possible risk factors include maternal diabetes, smoking hypertension during pregnancy, multiple gestations, and age [9]. ROP can lead to health complications, for instance, Retinal detachment, macular folds, and myopia [3, 6, 10]. In addition, ROP pulls its weight in causing developmental, social, and educational difficulties in ROP patients [11].

According to the international classification of ROP (ICROP), ROP can be classified into five stages: stage 1) has a white line between the vascular and avascular retinal junction, stage 2) is characterized by the presence of a ridge between the junction of the vascular and avascular retina, stage 3) is featured by the ridge proliferated into the extra retinal fibro vasculature area, stage 4) can be distinguished with subtotal retinal detachment, and stage 5) is marked by total retinal detachment [12].

1.2. ROP pathogenesis.

The development of retinal blood vessels begins at week 16 until they become fully vascularized by week 40 of gestation [13]. In case that infants are born prematurely, their retinal vascular system is not fully mature, exposing them to a greater risk of retinal diseases [14]. Angiogenic factors, such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)-1 have been demonstrated to be synergistically essential for the development of retinal vasculature. VEGF is an oxygen-dependent factor that is activated by hypoxia and inhibited by hyperoxia, conversely, IGF-1 is a non-oxygen-dependent factor that is found to be deficient in ROP patients [15-17]. ROP develops when retinal angiogenesis is altered by either deprivation of maternally derived factors

that drive normal evolution of retinal vessels or hyperoxic extra uterine environment [18]. ROP pathogenesis involves two phases: a vaso-obliterative phase and a vaso-proliferative phase [19].

The vaso-obliterative phase commences once the premature infant is born. In the uterus, the normal PaO2 level for the fetus is 30 mmHg, whereas, in the extrauterine environment, the Pa02 suddenly increases to 55–80 mm Hg [20]. This abrupt elevation in oxygen level suppresses hypoxia-driven angiogenic factors, namely VEGF, a paramount factor for retinal vessels growth. Consequently, arrest of vessels growth and retraction of already formed vessels transpire [18, 19]. These damaged vessels become hypoxic and unable to meet metabolic demands, leading to the initiation of the vaso-proliferative phase [21]. During this phase, in order to re-equilibrate with metabolic demand, these vessels upregulate the production of hypoxia-induced factors, particularly VEGF. In turn, VEGF engenders the formation of excessive vessels, however, these vessels fail to supply the avascular areas of the retina. Instead, they grow into the vitreous where they can cause bleeding, retinal detachment, and ultimately blinding [22].

ROP is also regarded as a neurodegenerative disease [23]. The hyperoxia exposure and switch from hyperoxia to hypoxia harm the retinal neurons as a result of oxidative stress. In addition, this switch triggers the release of free radicals, deficient blood supply, inflammatory response, and neuronal apoptosis [24]. Choroidal vessels play an important role in delivering oxygen and nutrients to retinal pigment epithelium and photoreceptors [25]. Degeneration of these vessels results in loss of RPE and degeneration of photoreceptors [26]. In line with these findings, > 25% choroidal thinning was observed in ROP patients using optical coherence tomography (OCT)[27]. Using the same test, the inner retinal layer was demonstrated to be thinner in ROP patients compared to control [28]. Multifocal Electroretinogram (mfERG) showed significantly shorter amplitudes and longer implicit times in ROP subjects than in healthy individuals [28]

1.3. Experimental models of ROP.

1.3.1. Oxygen-induced retinopathy (OIR) model.

OIR is a widely accepted method of studying vaso-obliteration and neovascularization in the retina [29]. The mouse OIR model was established by Smith et al. in 1994. In this model, postnatal day (P) 7-12 mice are exposed to 75% Oxygen. This hyperoxic condition resembles the hyperoxia that premature infants experience at birth [30]. During this course of hyperoxia, VEGF expression is suppressed. Resultantly, vaso-obliteration is instigated in the central retina within the first 48 hours, [31]. It is plausible that the occurrence of these changes is ascribable to the high nutritional demand in the developing retina [32]. Upon transferring animals into the room air at P12, these animals develop hypoxia, yielding ischemia in the vaso-obliterated areas. Consequently, VEGF is released in the ischemic regions [33]. Secreting VEGF activates the effector downstream molecules that participate in the formation of neovascular tufts. Then, neovessels start to form at the junction of the vascular and avascular areas, peaking by P17. Later, the vascular repairing process is commenced, featured with neovessels regression, normal revascularization, and limited proangiogenic production. Usually, this process finishes by P25 [34].

On the contrary to the OIR mouse model, the rat OIR model differs in the mode of hyperoxia induction and location of vascular changes. In mice, vaso-obliteration transpires in the central retina, whereas in rats, vaso-obliteration develops more peripherally [35]. In the rat OIR model, the newborn rats P0-P14 are exposed to an alternating approach of 50% oxygen (hyperoxia) and 10% oxygen (hypoxia), cycling every 24h [36]. Similarly, the OIR rat model can be induced by exposing rats to a seven-day cycle of gradual increase of O2 to 80% over 3 h, that is maintained for 20.5 h, followed by a rapid drop to 10% of O2, that is held for 0.5h. Then rats are returned to room temperature for 5 days [37]. Even though the rat OIR model mimics the human ROP, the

OIR mouse model produces more reproducible and robust means of induction of vaso-obliteration and neovascularization. In addition, the OIR mouse model can be genetically manipulated to provide transgenic mice for testing. Subsequently, the mouse model is more attractive in studying retinal vasculature development, and testing pharmacological efficacy in ischemic retinopathies [35]. In addition to the vascular damages, the OIR model exhibits neurodegeneration, gliosis, inflammatory changes, altered retinal function, etc [38-40].

1.3.1.1 Vascular changes in OIR

Normally, the mouse retinal vasculature starts to develop around birth and becomes fully mature by the third week after birth. Thus, mice at P7 are more susceptible to damage from hyperoxia as their retinas are still immature [41]. The high oxygen level causes vaso-obliteration by targeting the capillaries adjacent to arteries in the center of the retina, leaving capillary-free zones around these arteries [31]. Fascinatingly, larger, mature veins or arteries are not affected by hyperoxia as they are resistant to it. Therefore, developmental stages of retinal vasculature are more prone to hyperoxia [42]. A phenomenon that is explained by the fact that hyperoxia restricts the expression of VEGF, which in turn, reduces endothelial cell survival. On the other hand, the survival of the fully mature vasculature is independent of VEGF [43].

Paradoxically, exposing mouse pups to hyperoxia p7-p21 (with no return to room temperature at p12) results in a normal vascular regeneration without pathological neovascularization. This suggests that sudden onset of hypoxia is the major contributor of the pathological changes that bring about after returning animals to the room air, through enhancing the overexpression of hypoxia-inducible growth factor, resulting in upregulation of VEGF [42]. Hypoxia influences the retinal vasculature in different ways, including vascular sprouting, arterial tortuosity, and neovascular tufts [34]. In this regard, Vassey et al. illustrated that these blood vessels were absent

in the inner retinal, nevertheless, copious blood vessels were protruding outside the retina and into the vitreous [39]. VEGF upregulation has been strongly linked to the formation of neovascularization since studies have shown that inhibition of VEGF impedes neovascularization [44, 45].

VEGF is not the only element that drives the neovascular pathologies in the retina, other factors have been revealed to be implicated in these pathologies. To illustrate, astrocyte, a potent source of VEGF and crucially indispensable for vascular development, is lost during hypoxia [41]. Astrocyte loss occurs between P13-18, which mirrors the development of vascular pathology. Taking together, loss of astrocyte and proangiogenic factors may be responsible for the formation of neovascular tufts, and sprouting blood vessels into the vitreous [46]. This was supported by Michael et al. Michael and his group revealed that injecting astrocyte conditioned media intravitreally reversed the retinal neovascularization [47]. Besides astrocytes, inflammatory cells, including lymphocytes, microglia, and macrophages were associated with vascular pathology in the retina. A study aimed at blocking VEGF isoform 164 showed that VEGF influenced lymphocyte behavior in OIR [48]. Furthermore, using anti-inflammatory drugs reduced neovascularization severity [49]. Given that microglia are a known source of proangiogenic factors such as bFGF and VEGF, they have been linked to vascular pathology during OIR [50].

1.3.1.2. Neuronal changes in OIR

The neuronal circuit of the retina consists of rod photoreceptors that connect to rod bipolar cells, which in turn synapse with amacrine cells. Amacrine cells form gap junctions with ON cone bipolar cells and synaptic contacts with OFF cone bipolar cells [51]. OIR was demonstrated to decrease the number of retinal ganglion cells (RGC) by 26%, in comparison with control (room air) [52]. The investigations of neuronal retinal function in OIR using electroretinogram displayed

that a-wave (which indicates the activity of photoreceptors) is reduced. Also, the morphology and length of the rod's outer segment disc were disrupted [53]. Noteworthily, rod photoreceptors are affected more than cone photoreceptors. This is because rods develop after cones, which coincides with exposure to a high level of oxygen in OIR. As a result, hyperoxia disturbs rods development, eventuating in scotopic vision defect [54].

In addition to photoreceptor dysfunction, inner retinal neurons, particularly, amacrine cells are affected. This was corroborated by observing a reduction in b-waves (which is a reflection of post-receptoral function) after OIR [55]. Further, the structure of the retina is impaired. Downie et al. showed that in comparison with control, the inner nuclear retina was thinner in the OIR model. Narayanan et al. revealed that deletion of Arginase 2 in OIR mice preserved retinal thickness and inner nuclear layer, reduced rod bipolar loss, and improved retinal function [56]. On the molecular level, neuronal changes were noted in amino acids biochemistry as they are localized in the mammalian retina. Using the OIR rat model, glutamate was absent in all amacrine cells and avascular peripheral retina, while the central retina remained unaffected. Additionally, changes in glycine and GABA were noticed peripherally and centrally in both amacrine and bipolar cells. Specifically, glycine level was elevated, whereas GABA was lacking [57]. Activated microglia have also been shown to be implicated in neurotoxicity. An effect confirmed by the production of inflammatory and cytotoxic compounds like TNFa, and nitric oxide [58].

1.3.1.3. Glial changes in OIR

Microglia, astrocytes, and Muller cells (types of glial cells in the retina) are vital to support retinal neurons and maintain retinal function. They also play roles in energy metabolism and protection of the retinal blood barrier [59, 60]. Essentially, they release VEGF to stimulate vessels growth in response to hypoxia [61]. Gliosis was evident in OIR as it was demonstrated by the upregulation

of glial fibrillary acidic protein (GFAP) in Muller cells [62]. The expression of GFAP was observed in the peripheral and mid-peripheral avascular retina, suggesting that Muller cells are involved in retinal neovascularization in response to stress [59]. Furthermore, a high level of GFAP was associated with a reduction in the ability of Muller cells to maintain retinal fluid homeostasis, neurochemical metabolism, and the development of vasculogenesis [57, 63]. Consistent with these findings, Vassey et al. revealed that in the case of hypoxia, Muller cells become gliotic, attributing to vascular loss and neuronal dysfunction [39]. In a similar condition, Muller cells enhanced vascular permeability through HIF-dependent upregulation of the angiopoietin-like 4 (ANGPTL4) pathway [64]. Besides the involvement in vascular rarefaction, Muller cells-derived VEGF contributed to the development of retinal neovascularization. This was substantiated with a reduction in ischemia-induced retinal neovascularization in conditional VEGF knockout mice [65]. Clinically, tissues from patients with proliferative diabetic retinopathy showed that Muller cells-derived VEGF promoted expression of matrix metalloproteinase 2 (MMP2) in endothelial cells to induce retinal vaso-obliteration [66]. Collectively, these results indicate that Muller cells control retinal vascular integrity and vasculogenesis.

Microglia are also implicated in modulating neuronal and vascular changes in the retina [67, 68]. Microglia are present during the early development of the retina before even the formation of vascular plexus [68]. Removal of microglia during the formation of retinal vasculature interrupts retinal vascularization, yet, this was rescued with intravitreal injection of microglia [69]. The association of microglia with vascular pathology was demonstrated with a plethora of microglia in the retina, particularly, during neovascular tufts formation following OIR. This is pertinent to the ability of microglia to produce bFGF, metalloproteinases, and cytokines such as TNFα [50, 69, 70]. Exposing Sprague Dawley rats to hyperoxia-induced retinopathy showed vascular dropout,

microglial activation, and release of IL-1β. On the contrary, treatment with an IL-1 receptor blocker reversed these effects and promoted retinal revascularization [71]. Examining the effect of minocycline on vascular injury in the OIR model highlighted that despite its ability to reduce the reactivity of microglia and downregulate the proinflammatory mediators, minocycline failed to reduce retinal neovascularization and avascular areas, and worsen the visual function [72]. Moreover, the participation of microglia in vascular pathology entails cytokines, like MCP-1 and fractalkine [73]. In OIR, chemokine MCP-1 is upregulated [40]. Myriads of microglia are reduced in MCP-1 deficient mice following OIR [70]. Taking together, this implies that MCP-1 is required for the recruitment of microglia into the retina.

Morphologically, Vassey et al. showed that microglia had significantly larger soma areas with reduced processes following OIR [39]. In agreement with these findings, another study elucidated that amoeboid microglial cells were more in superficial central and mid-peripheral areas of the retina, alternatively, ramified microglial cells were fewer in these areas. Similar results were found in neovascular tufts areas [74]. As demonstrated by Fischer et al. instead of microglia density, activation of microglia reflects the involvement in neovascular tufts formation [74].

Concerning astrocytes, they are substantial for maintaining and developing retinal vasculature and neurons, upregulating antioxidants, and providing energy to neurons [75, 76]. Astrocytes have also been shown to release VEGF to facilitate the growth of vessels and endothelial cells during the early development of the retina. [21]. At this stage, astrocytes migrate from the optic nerve to the periphery of the retina [41]. The formation of vasculature in the retina is modulated by VEGF signaling, and platelet-derived growth factor-A, secreted by both astrocytes and RGCs, respectively. In support of this, blocking the action of PDGF-A in the eye lowered the number of astrocytes, whereas overexpressing PDGF-A in RGCs increased the number of astrocytes. Taken

together, this unequivocally proves the involvement of RGCs and astrocytes in retinal vascular pathology [77]. In the OIR model, astrocytes were degenerated during the hypoxic phase, thereby causing blood-retinal barrier disruption [78]. This absence of astrocytes halted vascular development and engendered vascular pruning in the retina [79]. Additionally, Bucher et al. found that the density of astrocytes was reduced and this reduction was observed during the hyperoxic phase between P8-P10. These apoptotic astrocytes were displayed in the central avascular area, however, no astrocyte loss or reduction in density was observed in vascular tufts [80]. Accumulating evidence reported depletion of astrocytes within the hypoxic phase [47, 81, 82].

Injection of astrocytes conditioned media promoted revascularization and reduced retinal neovascularization in OIR [47]. The inhibition of the renin-angiotensin system (RAS) by Valsartan presented a retinoprotective effect through abolishing astrocytes loss [46]. Further, deletion of soluble epoxide hydrolase, an enzyme that metabolizes docosahexaenoic acid (DHA) and controls retinal vasculogenesis and vascular balance, resulted in peripheral avascular areas, central neovascular tufts formation, and astrocytes death. Conversely, treatment with 19,20-dihydroxydocosapentaenoic acid (19,20-DHDP) rescued the aforementioned pathological changes in OIR model [83].

1.4. Mechanisms underlying vascular damage in ROP

1.4.1. Oxygen-dependent factors

1.4.1.1. Hypoxia-inducible factor (HIF-1α)

HIF-1 α is an oxygen-dependent subunit of HIF-1 that regulates angiogenesis beyond its other cellular functions. Normally, HIF-1 α is broken down by 26S proteasome[84]. However, In case of hypoxia as in the OIR model or once supplemental oxygen is removed from premature infants,

HIF-1α degradation is inhibited by prolyl hydroxylase, and hence allows the transcription and release of the angiogenic factors, for instance, VEGF, angiopoietins, and erythropoietin [85]. Experimentally, in mice, studies have reported that administration of HIF-1α -induced growth factors during hyperoxic phase 1 reduced loss of vasculature, contrarily, inhibition of HIF-1α in hypoxic phase 2 averted neovascularization [86, 87].

1.4.1.2. VEGF

VEGF is a salient growth factor for the maturation of retinal vasculature, produced by different cell types in the retina [88]. During phase 1, VEGF is suppressed by hyperoxia, resulting in the arrest of normal vascular development in neonates. In the mouse model of oxygen-induced retinopathy (OIR), VEGF mRNA level was diminished 6 h after exposure to 75% oxygen, provoking vessels loss [88, 89], demonstrating that suppression of VEGF is a major contributor to vessels loss in phase 1 [90]. On the contrary, in phase 2, the VEGF level increases once high oxygen exposure is lifted, generating neovascularization [91]. This was confirmed experimentally when the administration of anti-VEGF therapy restrained neovascularization in the OIR model [92].

1.4.1.3. Erythropoietin (Epo)

Epo is another oxygen-dependent growth factor that has been elucidated to be involved in retinal angiogenesis [18]. In the OIR model, Epo protein is expressed in retinal vessels and inner retina at P8, and its expression is regulated by HIF-1α [93, 94]. In phase 1 of the OIR mouse model, mRNA expression of Epo was decreased, suggesting that reduction of Epo level could attribute to vessels loss. Moreover, the administration of Epo in phase 1 was shown to be vasoprotective through the restoration of damaged vessels and protection against neuronal damage in the retina [93]. This advantageous effect is explained by inhibition of caspases and recruitment of endothelial

cells from bone marrow to the retina [93]. In contrast, in phase two of the OIR model, Epo was markedly high during neovascularization; however, inhibition of Epo in this phase deterred the vaso-proliferation.[95].

1.4.2. Oxygen independent factors

1.4.2.1. Insulin-like growth factor-1 (IGF-1)

IGF-1 is a crucial growth factor for fetus growth and development during gestation. IGF-1 was found to be depleted in premature infants, which has been hypothesized to be a risk factor for ROP [96]. In this regard, Vessel loss was observed in IGF-1 knockout mice, demonstrating the importance of IGF-1 in angiogenesis. Captivatingly, in the same study, levels of VEGF were similar to those in wild-type IGF-1, suggesting that VEGF solely is not sufficient to promote vasculogenesis [97]. Additionally, in the OIR mouse model, treatment with IGF-1 antagonist lessened neovascularization, an effect that was manifested by inhibition of VEGF-mediated mitogen-activated protein kinase pathway (MAPK), necessary for the survival and proliferation of endothelial cells [98]. This unequivocally indicates that IGF-1 plays a role in mediating VEGF-induced vessels growth [99].

1.4.2.2. ω-3 Polyunsaturated Fatty Acids (PUFAs)

Like IGF-1, PUFAs transfer from mother to fetus during the third trimester to support neuronal and vascular growth [18, 100]. In the case of prematurity, PUFAs are diminished, halting vascular and neuronal growth. This was corroborated by a reduction in vaso-obliteration in newly born pups treated with 2% of total fatty acids from ω-3 PUFAs through inhibition of inflammatory cytokines [101]. Equally, exogenous treatment with ω-3 PUFAs restricted both vaso-obliteration and retinal neovascularization [102].

1.4.2.3. Placental growth factor (PIGF)

PIGF is a growth factor that is vital for vasculogenesis. Unlike VEGF, PIGF level was reduced under hypoxic conditions in both OIR mice and CoCl2-induced hypoxia in human umbilical vein endothelial cells (HUVECs). Intriguingly, PIGF expression increased with anti-VEGF treatment (Bevacizumab) [103]. In addition, PIGF level was found to be remarkably upregulated in aqueous humor, and vitreous of patients with ischemic retinopathies [104, 105]. Alternatively, pharmacological inhibition or deletion of PIGF was shown to be effective in ameliorating retinal leakage, neovascularization, inflammation, and gliosis [106-108].

1.4.2.4. Angiopoietin 1 and 2 (Ang1 and Ang2)

These are other growth factors that are imperative to promote angiogenesis and maintain vessels stability. Both are ligands for the Tie2 receptor [109]. Studies have revealed that Ang1 preserves vessels stability, while Ang2 mediates vaso-proliferation [109, 110]. Besides, Ang 2 level was found to be high in the vitreous of eyes with severe ROP [111]. Retinal angiogenesis was remarkably reduced with inhibition of both Tie2 and VEGF receptors compared to the VEGF receptor alone [109].

1.4.3. Inflammatory factors in ROP

Recent studies have indicated that prenatal and postnatal inflammation contribute to ROP, however, the role of inflammation in ROP remains ill-defined [112]. Cytokines and chemokines are inflammatory mediators secreted by immune cells and were demonstrated to be implicated in the development of ROP [113].

1.4.3.1. Cytokines

Following hypoxia, microglia release interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), both of which were shown to be egregious to the retina [114]. In the OIR model, IL-1β has been

involved in retinal microvascular deterioration and blood vessels shrinkage [26, 71]. On postnatal days 0-3, high levels of IL-6 and C-reactive protein were detected in patients with ROP compared to control [115]. Likewise, Analysis of eyes with ROP revealed high levels of several cytokines, including IL-6, IL-7, IL-10, IL-15, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, etc. [116]. Another study reported that COX-2 contributed to retinal neovascularization through the production of prostaglandin E2 (PGE2) [117].

1.4.3.2. Chemokines

Chemokines control the migration of microglia into the inflammation site. Among chemokines implicated in ROP are IL-8, Regulated on Activation, Normal T Expressed and Secreted (RANTES), monocyte chemotactic protein 1 (MCP-1), and interferon-inducible T-cell α chemoattractant (I-TAC) [113]. In humans, the IL-8 level was elevated in premature infants with ROP, while in rats, the IL-8 level peaked during neovascularization [118, 119]. Similarly, MCP-1 level was noticed to be higher in premature infants with ROP compared to healthy infants [120]. In the animal model of ROP, MCP-1 triggered retinal neovascularization by attracting macrophages [40].

1.4.4. Nitro-Oxidative stress

The involvement of oxidative stress in ROP is owed to the vulnerability of the retina to reactive oxygen species (ROS), which results from exposure to oxygen fluctuation [121]. Noteworthily, these species can act as signaling molecules to induce either physiological or pathological processes. ROS can trigger endothelial dysfunction. For instance, the activation of nitric oxide (NO) by nitric oxide synthase (NOS), specifically, endothelial NOS (eNOS), can act as a blood vessel relaxing agent. Yet, in phase 1 OIR, it can forms peroxynitrite, engendering microvascular deterioration. Furthermore, oxidative stress has been illustrated to activate VEGF receptor 2

(VEGFR2) signaling in phase 2 of the OIR model [122]. Reciprocally, VEGF was shown to increase the production of ROS through activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) in endothelial cells [123]. Adopting the same animal model, both eNOS and inducible NOS (iNOS) were indicated to mediate retinal neovascularization [124, 125]

ROS can also be generated by NADPH oxidase, which has been reported to cause vascular pruning in OIR phase 1 and vaso-proliferation in phase 2 through activation of NOX 1, NOX2, or STAT3 [126]. ROS including hydrogen peroxide (H2O2), singlet oxygen (O2), superoxide anion (O2−), and hydroxyl radical (OH) are mainly produced by mitochondria and NOX. NOX members are pivotal in cell transduction, migration, and proliferation [127]. Further, NOX was revealed to be a major source of ROS and engaged in retinal neovascularization through activation of the JAK/STAT signaling pathway. This was substantiated by the amelioration of neovascularization via inhibition of NOX and JAK/STAT signaling pathways [128-130]. NOX inhibition not only mitigated neovascularization, but also reduced vessel loss in the early phase [131]. In mouse retinal endothelial cells, NOX1 and NOX4 appear to be the most prevailing isoforms that are expressed following stress [132, 133]. Consistent with these findings, NOX1 and NOX4 were detected via DHE staining in the retinas of OIR mice, suggesting that hypoxia enhances the generation of ROS. ROS can also promote inflammation through the activation of caspase-1 and pro-inflammatory cytokines. The same study found that a high level of ROS induces NLRP3-caspase-IL-1β, facilitating inflammasome activation [134]. In this line of evidence, Sui et al. proclaimed that inhibition of NLRP3 inflammasome with MCC950 lessened retinal neovascularization through opposing IL-1β/IL-18 activation [135]. Moreover, oxidative stress has been demonstrated to increase heme oxygenase-1 (HO-1), a factor that is cytoprotective against oxidative stress [136]. During the first week of life, the expression of HO-1 was found to be high in premature infants

[137]. In parallel, oxidative stress-induced the expression of HO-1 in muller cells of the mouse retina [138]. Mechanistically, in an immature retina, ROS interacts with NO to produce reactive nitrogen species (RNS), in particular, dinitrogen trioxide peroxynitrite, and nitrogen dioxide [139]. These compounds impair the vasculature [140, 141]. Another mechanism that contributes to nitrative stress is the elevation of CO2. Despite this debated mechanism, hypercapnia has been proven to induce microvascular deterioration in vitro [142].

Lipid peroxidation has also been linked to retinal diseases through the production of peroxidases, which further increases the generation of thromboxane 2 (TXA2) [143]. TXA2 is a vasoconstrictor and cytotoxic to blood vessels that has been shown to be involved in inducing endothelial cell death in the ROP animal model [144]. Following along with this line of evidence, treatment of mice with indomethacin, a TXA2 synthase inhibitor, alleviated retinal vaso-obliteration [144]. During lipid peroxidation, platelet-activating factor (PAF) and lysophosphatidic acid (LPA) are generated, both of which are known mediators of microvascular injury [145, 146]. Besides, cis- to trans-isomerization of arachidonic acid (TAA) had been claimed to engender nitrative stress, provoking retinal vascular damage in the OIR mouse model [147]. It is conceivable that this detrimental effect of TAA is due to the formation of thrombospondin-1[148]. Nonetheless, in eNOS deficient mice and mice treated with NOS inhibitor, TAA production was abolished [148].

Nuclear erythroid 2-related factor 2 (Nrf2) is a transcription factor that acts as a defensive mechanism opposing oxidative stress [149]. While age-related retinal degeneration was apparent in Nrf2 knock-out mice [150], a study found that up-regulation of Nrf2 protected retinal ganglion cell (RGC) death from oxidative stress in nerve crush (NC) animal model [151]. Moreover, Nrf2 played a protective role in animal models of diabetic retinopathy and ischemia-reperfusion injury [152, 153] As a result of these findings, numerous Nrf2 activators were tested for their usefulness

in retinal diseases, still, the clinical efficacy of these agents is not practically feasible[154]. Additionally, N-acetyl cysteine, vitamin E, and lutein were examined in clinical trials to assess their effectiveness as antioxidants for the treatment of retinopathy of prematurity. Notwithstanding, these agents failed to prove their effectiveness, rather, they were associated with unfavorable side effects [155-157].

Previous studies from our lab have demonstrated the role of the arginase/polyamine oxidase signaling pathway in OIR induced neuronal and vascular injuries [56, 158]. Deletion of Arginase 2 (A2) protected against hyperoxia induced neurodegeneration and vascular damage [56]. Our studies demonstrated for the first time that A2 deletion significantly improved neuronal survival and function, through mechanisms involving the regulation of mitochondrial membrane permeability mediated apoptosis during retinal ischemia. Further studies demonstrated the critical role of polyamine oxidation and spermine oxidase (SMOX, enzyme involved in the catabolism of polyamines) in hyperoxia induced neuronal damage and vaso-obliteration [158, 159]. Treatment with SMOX inhibitor reduced OIR induced endothelial cell death, microglial activation, and levels of inflammatory molecules, suggesting the potential cross-talk between glia, neurons, and vasculature [159]. However, the specific role of SMOX or the molecular mechanism regulated by SMOX in mediating OIR-induced vascular damage is yet to be elucidated.

1.5. Current treatments, challenges, and need for novel therapies.

The recommended therapies for ROP are anti-VEGF, laser therapy, and cryotherapy.

1.5.1. Cryotherapy

Cryotherapy emerged in the 1980s as a first treatment strategy for ROP, however, at that time no guidelines were available indicating the use of cryotherapy based on the disease severity [160]. Given that, the Cryotherapy for Retinopathy of Prematurity (CRYO-ROP) study was conducted in the mid-1980s with follow-up for up to 20 years to determine when to use cryotherapy. The results of this study revealed that cryotherapy is effective in treating stage 3 ROP with a remarkable improvement in retinal detachment, and formation of retrolental fibrous tissues [161]. Despite these benefits, cryotherapy was linked with some unpleasant outcomes including, inflammation, visual acuity, loss of visual field, and myopia [162-165]. These outcomes have made it a less favorable treatment for ROP since the advent of laser therapy [166].

1.5.2. Laser therapy

Another treatment for ROP is laser photocoagulation, which works by upregulating pigment epithelium-derived factor. This factor can trigger cell death in activated vascular endothelial cells and preclude neovascularization [167]. Laser therapy has been endorsed over cryotherapy since it has fewer systemic complications, and does not necessitate general anesthesia. [168]. Laser photocoagulation is recommended for stage 1 or 2 ROP patients, with the advantage of having complete retinal revascularization 3 to 4 months following the treatment [169]. Studies have demonstrated that laser therapy had better visual acuity outcomes compared with cryotherapy [165, 170, 171]. The Early Treatment of ROP (ETROP) study was conducted to evaluate the effectiveness of laser therapy as an early intervention in ROP. The results of this study showed that both negative visual and anatomical outcomes were lowered from 19.8% to 14.3%, and from

15.6% to 9.0%, respectively [172]. Despite the great advantages of laser photocoagulation, it has some adverse effects, specifically, reduction in visual field and night vision, myopia, and impaired dark adaptation [173, 174]. Also, taking into consideration the destructive nature of laser therapy, the damage to retinal cells cannot be disregarded [175]. For these reasons, clinicians shifted towards anti-VEGF therapy [173]

1.5.3. Anti-VEGF therapy

Controlling the expression of VEGF in ROP can bring back physiological hemostasis and allow the formation of intraretinal vascularization, thereby preventing pathological angiogenesis [176]. As of now, the available anti-VEGF agents in use are Bevacizumab, Pegaptanib, Ranibizumab, and Aflibercept. Unlike laser therapy, VEGF inhibitors do not ablate the peripheral retina to diminish VEGF production, instead, they bind to the VEGF receptor and cease its action [177]. Thus, VEGF inhibition appears to be an ideal treatment for ROP since it reduces the necessity for invasive therapies, like cryotherapy or laser, and restores the normal physiological level of VEGF [166].

The Bevacizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity study (BEAT-ROP) revealed that Bevacizumab minimized the ROP recurrence rate to 6% compared to 42% in the laser therapy group. Intravitreal anti-VEGF treatment ceased the progression of ROP in over 90% of patients [178]. In a five-year follow-up study, no evidence of abnormal MRI findings or systemic side effects attributable to bevacizumab therapy was found [179]. Another 41 weeks follow-up study of combination therapy of anti-VEGF and laser photocoagulation was shown to be more effective with neither retinal detachment nor recurrence [180]. Further, the need for retreatment with anti-VEGF therapy was reported in only 4% compared to 22% in laser therapy, indicating the efficacy of anti-VEGF in treating ROP [181]. The risk of myopia was only 2.7% in

patients treated with anti-VEGF compared to 42% in the laser therapy treated group [182]. While some studies have revealed that ranibizumab, bevacizumab have similar efficacy, deciding which one is the drug of choice is still under study [173]

Despite the auspicious efficacy of anti-VEGF, there are some substantial concerns. For instance, Anti VEGF medications are expensive and this limits their use in clinical settings [183]. VEGF plays a salient role in maintaining homeostasis of glia, and neurons and developing retinal vasculature, kidney, and lung. Hence, inhibiting VEGF can chaotically influence the physiological or desired effect of VEGF in the body [88, 184-186]. This is a serious issue since a single dose of anti-VEGF can reduce VEGF levels for up to 2 months.[187]. Natarajan et al. illustrated that treatment with bevacizumab resulted in a high mortality rate and impaired cognitive outcomes compared to other therapies [188]. Equally, treatment with anti-VEGF is associated with high risks of developing hypertension and cardiovascular events [189]. Inhibition of VEGF does not control the expression of other angiogenic factors, such as angiopoietins or erythropoietin, both of which are well known to promote recurrent neovascularization due to their up-regulation [190].

There are challenges with the current treatment of ROP. The limitations associated with the anti-VEGF therapy, need further attention. The time of anti-VEGF administration, the impact of VEGF blocking on retinal ganglion cells since VEGF is expressed in neurons, the long-term effect of VEGF inhibition on retinal function, the cost-effectiveness comparison with other treatments, and the systemic effect of anti-VEGF on the human body are some of the major concerns [14].need to be considered in the clinical trials. It is also worth mentioning that considering the complexity of ROP pathogenesis, a more profound understanding of the interplay between neuronal, vascular, and glial changes is mandated to unravel optimal therapies for ROP.

Problem Statement and Central Hypothesis

Ischemic retinopathies including retinopathy of prematurity and diabetic retinopathy are the principal causes of blindness among children and working-age adults. These retinal diseases are characterized by microvascular injury, neurodegeneration, glial changes and inflammatory response. For decades, these were considered purely as vascular pathologies and the existing current therapies including laser photocoagulation, cryotherapy, and anti-VEGF, only treat the advance stages of retinopathy and are associated with harmful side effects. Recent studies demonstrate that glial, neural, and microvascular dysfunctions in the retina are interdependent and intimately involved in the development of ischemic retinopathies. Hence, there is a great need to understand the interplay between neuronal, vascular, and glial changes in retinal diseases to develop a novel therapeutic alternative. Spermine oxidase (SMOX) is an enzyme in the polyamine metabolic pathway that oxidizes spermine into spermidine, playing a prominent role in polyamines hemostasis. Clinically, SMOX levels were shown to be altered in insulin-dependent diabetic patients. Pre-clinically, using the experimental models of ischemic retinopathies (OIR and streptozocin (STZ)-induced diabetic retinopathy), our laboratory was the first to show the implication of SMOX in mediating retinal neuronal injury. In agreement with these findings, inhibition SMOX using MDL 72527 reduced excitotoxicity, diabetes and hyperoxia-induced retinal neurodegeneration. Despite this growing body of evidence, the exact role of SMOX on causing vascular injury in ischemic retinopathy is still unexplored. Accordingly, the **objective** of our study is to investigate the impact of neuronal spermine oxidase on vascular damage in a model of ischemic retinopathy. Our **central hypothesis** is that activation of neuronal SMOX aggravates vascular injury in ischemic retinopathy.

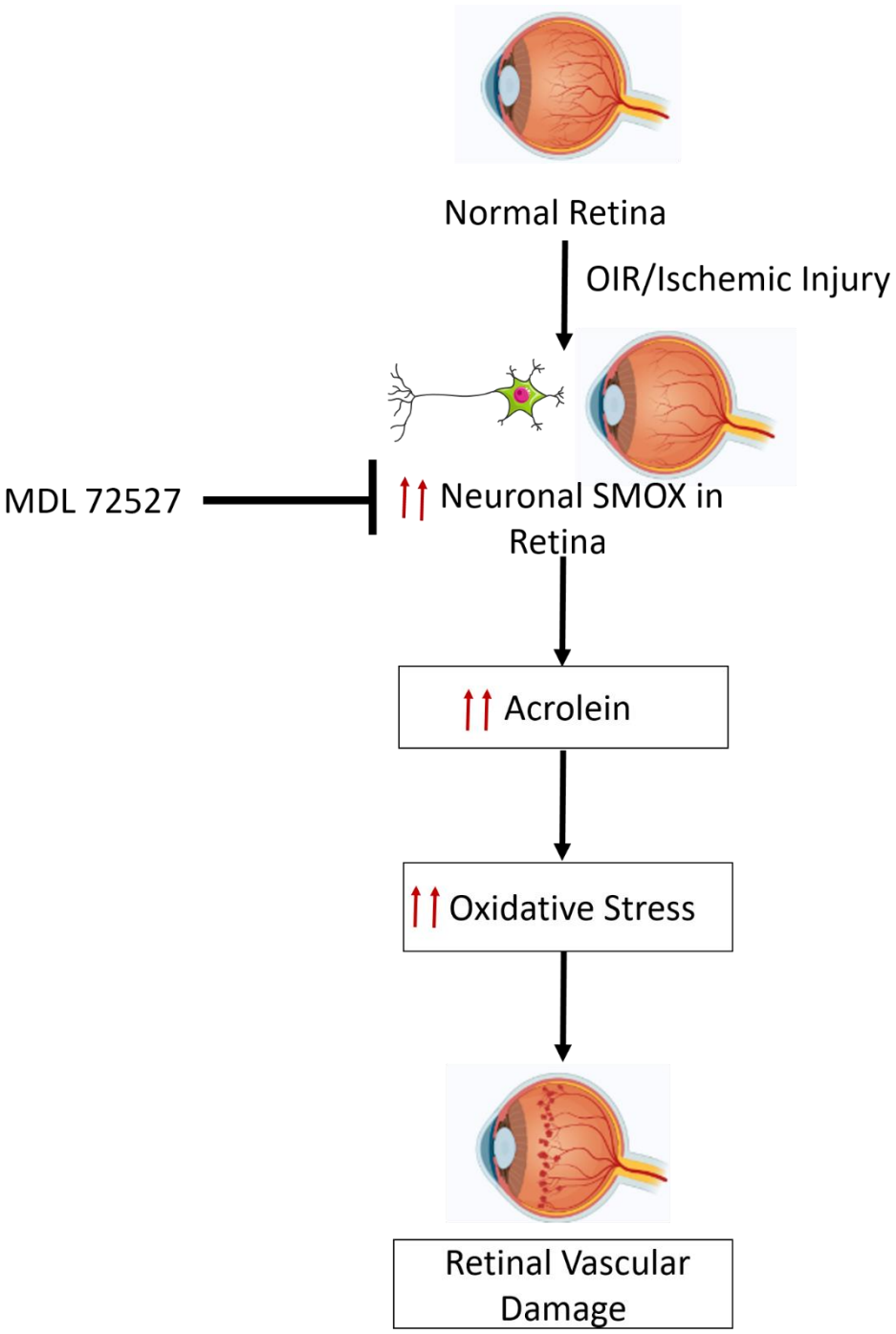

Figure.1. Schematic diagram of the central hypothesis. Activation of neuronal SMOX aggravates vascular injury in ischemic retinopathy.

Specific Aims

We propose to test our hypothesis by studying the following specific aims:

Aim 1: Investigate the impact of neuronal SMOX on retinal vascular damage in a model of ischemic retinopathy.

These studies will be performed utilizing the oxygen-induced retinopathy (OIR) model, transgenic mice overexpressing SMOX in retinal neurons (SMOX tg) available in our laboratory and treatment (in vivo) with SMOX inhibitor, MDL 72527. Retinas will be isolated, flat-mounted, and immunostained with Isolectin B4 and GFAP to examine the effect of SMOX and its inhibition on the loss of blood vessels, neovascular tufts formation, and gliosis.

Aim 2: Investigate the cell survival and cell death signaling pathways regulated by SMOX in ischemic retinopathy.

To accomplish this aim, Western blot studies will be employed to study the changes in survival /stress signaling molecules and oxidative stress/antioxidant markers.

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CHAPTER 2

Role of Neuronal Spermine Oxidase in Vascular Damage in a Model of Ischemic Retinopathy[1]

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To be submitted to the Journal of Clinical Medicine

Abstract

Retinal vascular injury is a major cause of vision impairment in ischemic retinopathies such as diabetic retinopathy and retinopathy of prematurity (ROP). Current treatments for ischemic retinopathies target the late stage of the disease and have long-term side effects. Therefore, there is a great need to identify novel mechanisms and therapeutic approaches. Previous studies from our laboratory have shown the critical role of spermine oxidase (SMOX) in retinal neurovascular damages. Utilizing the oxygen-induced retinopathy (OIR) mouse model of ROP, the current study was undertaken to investigate the impact of neuronal SMOX in mediating retinal vascular injury. Newborn mice (WT control, SMOX Tg, or WT treated with MDL 72527/saline) along with their mother were maintained in 70% oxygen from postnatal day (P) 7 to 12 followed by normoxia until P17. Animals were sacrificed at P17 and eyes were processed for different experiments. Our data demonstrated that retinal vascular development was normal in the SMOX Tg retina, and MDL 72527 treatment had no deleterious effect on WT retinas. Staining of retinal flat-mounts with Isolectin B4 showed that SMOX overexpression significantly increased OIR induced avascular area and pathological neovascular tufts in SMOX Tg retinas compared to WT-OIR retinas. Treatment with SMOX inhibitor, MDL 72527, significantly reduced these pathological changes. Furthermore, Glial fibrillary acidic protein (GFAP) immunostaining of retinal cryostat sections showed elevated glial activation in SMOX Tg-OIR retinas compared to WT-OIR retinas. Western blot analysis of retinal lysates performed on OIR groups treated with MDL 72527/vehicle demonstrated increased levels of acrolein conjugates compared to normoxia control groups. Considering the urgent need to identify new therapeutic modalities for retinal neovascular diseases, our data suggest that blockade of SMOX signaling can be a potential therapeutic target to limit the progression of ischemic retinopathies.

Introduction

Ischemic retinopathies (IR) such as diabetic retinopathy (DR), retinopathy of prematurity (ROP) have been the leading cause of vision loss and impairment in adults, and children, respectively [1, 2]. These pathologies are associated with various features including microvascular damage, inflammatory response, and neuronal death [3]. Despite the different triggers in each of these diseases, they are characterized by similar characteristics and stages of progression [4]. The retinal vascular damage in IR is biphasic, with the initial phase characterized by vessel deterioration [5]. In the subsequent phase, these damaged vessels become ischemic, precipitating in compensatory neovascularization, and consequently leading to retinal detachment and blindness [6].

The interplay between glial, and endothelial cells, and ganglion cells is vital for retinal vascular growth [7]. The normal vascular development of the retina is firmly controlled by the balance between pro-angiogenic and angiostatic factors [8] released by neurons and glia. In case of pathological neovascularization, an insult like hypoxia, ischemia, or inflammation disrupts this balance in the retina, causing elevated levels of VEGF excessively through activation of HIF-1 [8]. Retinal neovascularization is associated with the breakdown of the inner membrane, permitting sprouting of new vessels into the vitreous and thereby causing fibrosis and retinal detachment [9]. Typically, the endothelial cells of the new retinal blood vessels contain tight junctions that preserve (BRB) [10]. Yet, in pathological neovascularization, these vessels become fragile owing to the disruption of the tight junctions, leading to high vascular permeability and eventually retinal vascular leakage [10]. Furthermore, Muller cell is a key factor in maintaining the integrity of the blood-retinal barrier (BRB). In the normal condition, Muller cell secretes pigment epithelium-derived growth factor (PEDF), which is required to counteract VEGF action [11]. However, under hypoxia, PEDF expression is lowered, resulting in unrestricted VEGF action

and hence high vascular permeability [12]. The integrity of BRB is also maintained by pericytes and retinal glia, both of which were found to be lost in pathological neovascularization, aggravating vascular damage [13].

Despite the extensive research conducted on understanding the pathogenesis of pathological retinal neovascularization, the exact mechanisms are not completely studied. Understanding the molecular regulators of retinal vascular damages will facilitate the development of new approaches to restore normal retinal vasculature. The current therapies for IR, including laser photocoagulation, cryotherapy, and anti-VEGF treatments, treat only the advanced stages of the diseases and are associated with harmful side effects. Thus, there is a great need to understand the novel mechanisms including the interplay between neuronal, vascular, and glial changes in the pathogenesis of ischemic retinopathies to develop more effective and promising therapies.

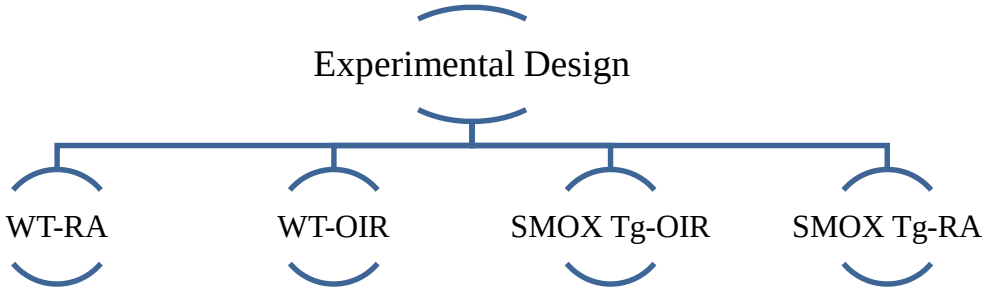
Polyamines (spermine, spermidine, and putrescine) are essential for cell growth, differentiation, and apoptosis [14, 15]. Polyamine metabolism is regulated by Spermine Oxidase (SMOX), Spermine Spermidine Acetyl Transferase (SSAT), and Acetyl Polyamine Oxidase (APAO) [16]. Dysregulation of polyamines metabolism has been reported to be implicated in neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, ischemic brain damage, traumatic brain injury, and death of retinal ganglion cells [17-23]. SMOX is an enzyme that oxidizes spermine into spermidine and plays a critical role in polyamines hemostasis [24]. During this oxidation hydrogen peroxide (H2O2) and aldehyde 3-aminopropanal (3-AP), are produced, both of which are initiators of cellular damage [16]. The byproduct, 3-AP can be converted into a highly reactive aldehyde called acrolein, a known mediator of inflammation, DNA and protein adduction, and membrane disruption [25]. Our laboratory was the first to show the critical involvement of SMOX in causing retinal neuronal injury [16]. This was apparent by an elevation in SMOX level,

and alteration in polyamines levels in the mouse model of hyperoxia-induced neuronal degeneration. In agreement with these findings, treatment with N1, N4-bis (2,3-butadienyl)-1,4-butane diamine (MDL 72527), a polyamine oxidase inactivator reduced hyperoxia-induced degeneration of retinal neurons [26]. Additionally, inhibition of SMOX prevented retinal neurodegeneration in models of retinal excitotoxicity and diabetic retinopathy [27, 28]. Studies from others have demonstrated that upregulation of SMOX mediates ROS formation [29, 30]. Clinically, SMOX levels were shown to be altered in insulin-dependent diabetic patients [31], and altered polyamines levels are observed in vitreous samples of proliferative diabetic retinopathy patients [32]. However, not much is known about the direct impact of SMOX in causing retinal vascular damage in ischemic retinopathy. Considering the fact that ischemic retinopathies are neovascular diseases, investigating the role of neuronal SMOX in causing vascular injury in ischemic retinopathy would provide novel insights into the molecular mechanisms. Utilizing the well-established Oxygen Induced Retinopathy (OIR) of IR, this study was undertaken to investigate the impact of neuronal SMOX on retinal vascular damage.

Methods and materials:

Animals and the OIR model.

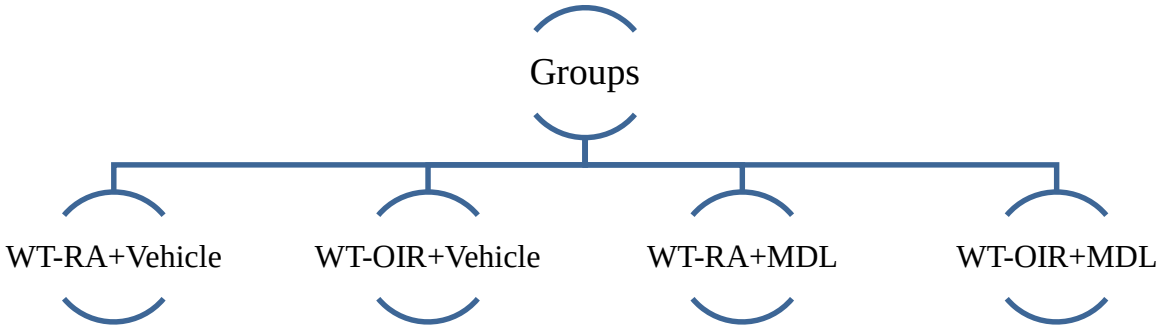
All animal procedures were approved by Institutional Animal Care and Use Committee (IACUC) at Charlie Norwood Veteran Affairs medical center (CNVAMC), Augusta, GA. In this study, efforts were made to minimize animal suffering. Both wild-type (WT, C57BL/6J) control and SMOX Tg (transgenic mice overexpressing SMOX in retinal neurons, available in our laboratory) mice were maintained in the animal facility at CNVAMC. The experimental model of OIR was induced according to the methods standardized in our laboratory [26]. Briefly, postnatal day (P7) newborn mice along with their nursing mothers were placed in a 70% oxygen chamber (hyperoxia phase) till P12, then, mice were returned to room air and maintained until P17 (hypoxic phase) [128]. In the present study, animals were divided into either room air or OIR groups as shown below. WT-room air (RA), WT-OIR, SMOX-RA, and SMOX-OIR. Mice were euthanized at different time points between P12 to P17, and eyeballs or retinal tissues were collected and analyzed as described below.



Schematic diagram-1. The experimental design of oxygen induced retinopathy experiment. Animals in this experiment were divided into four groups, WT-RA, WT-OIR, SMOX-RA, and SMOX-OIR.

MDL 72527 treatment.

MDL 72527 (M2949, Sigma, St. Louis, MO, USA) was injected intraperitoneally with a daily dose of 40 mg/kg/day in 0.9% saline from P12 to P16. Vehicle (0.9% saline) treated animals were used as a control in this experiment. Animals were divided into WT-RA, and WT-OIR treated with vehicle, and WT-RA treated with MDL 72527 and WT-OIR treated with MDL 72527.



Schematic diagram-2. The experimental design of oxygen induced retinopathy experiment and MDL 72527 treatment. Animals in this experiment were divided into four groups, WT-

Isolation, flat-mounting, and immunostaining of retina.

Mice were deeply anesthetized (ketamine/xylazine 0.01 ml/g of body weight) and the eyes were enucleated at p17. Eyeballs were fixed in 4% paraformaldehyde (overnight) and stored in PBS. Eyeballs were then carefully dissected, cornea, lens, sclera, and vitreous were removed and retinas were cut into four quadrants to be flattened. Retinas were blocked with a blocking buffer, containing 1% Triton-X-100 and 10% donkey serum for 30 min and incubated with Isolectin B4 (Invitrogen, Carlsbad, CA, USA) overnight at 4 °C with gentle rocking (at 1:150 dilution in blocking buffer). On the following day, retinas were washed (using PBS, 30 min each) three times

and flattened on a glass slide using a mounting medium (H-1500, Vector Libroratories, Inc, Burlingame, CA, USA).

Imaging, and quantification of avascular areas and neovascular tufts formation.

The immunostained retinal flatmounts were used for image acquisition. Several 10X images were taken using the BZ-X800 Keyence fluorescence microscope (Keyence Corporation of America, Itasca, IL, USA), and images stitching was executed, and the images were saved in Tiff format. Quantification of the area of neovascularization was performed as the published methods by SWIFT-NV-step by step quantification method, installed on ImageJ software (National Institute of Health, MD, USA) [33]. After determining the appropriate fluorescence intensity threshold of vascular tufts manually by the user, the neovascular area in the four different quadrants over the total retinal area was calculated. To measure the avascular area, regions without vessels growth were manually selected and measured in ImageJ and divided by the total area of the retina.

Preparation of retinal cryostat sections, immunostaining, and imaging.

The eyeballs were fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, and cryoprotected by placing in 30% sucrose (M-12681, Fisher Chemical, Fair Lawn, NJ, USA) for three days at 4°C with gentle rocking. Eyeballs were then embedded in Tissue-Tek optimal cutting temperature (O.C.T) compound (4583, Sakura Finetek, Torrence, CA, USA) and cryostat sections were prepared at the Augusta University histology core. Retinal sections (10 μm) were brought to room temperature and washed once with PBS for 10 min. Small boundaries around the retinal sections were drawn using ImmEdge pen (NC9545623, Vector Laboratories, Inc. Burlingame, CA, USA). Sections were blocked (10% NGS and 1% Triton-X-100) for 1 h at room temperature and incubated overnight with primary antibody (Glial fibrillary acidic protein (GFAP) 1:500) (Z0334, Dako Agilent, Santa Clara, CA, USA) at 4°C. On the next day, sections were washed with PBS

three times (10 min each) followed by incubation with Donkey anti-Rabbit IgG Highly Cross-Adsorbed Secondary Antibody, (Alexa Fluor 555, A-31572, Thermofisher Scientific, Waltham, MA, USA) 1:200 for 2 h at room temperature. The sections were washed with PBS three times, 10 min each, and cover slipped with mounting medium.

Image acquisition was performed using Zeiss 780 Upright Confocal microscope (LSM 780, ZEISS Research Microscopy Solutions, White Plains, NY, USA) at 20x magnification, located at the imaging core facility, Augusta University. Two channels were used to take the images, 4′,6-diamidino-2-phenylindole (DAPI, 405 laser power), and red fluorescent protein (RsRFP, 543 laser power). Quantification of fluorescence intensity was performed using Image J by dividing the total fluorescence intensity by the background fluorescence intensity of the selected area.

Western blotting

Retinas were homogenized in RIPA lysis buffer (20-188, EMD, Millipore Corp, Billerica, MA, USA) supplemented with protease and phosphatase inhibitors (78430, Thermo Scientific, Rockford, IL, USA). The lysates were centrifuged at 14.000 revolutions per minute (RPM) for 20 minutes (Centrifuge 5430 R, Eppendorf, Farmingham, MA, USA) and the supernatants were collected. Protein estimation was done using the bicinchoninic acid kit (Pierce, Thermo Scientific). Around 25ug of protein per sample was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) (1620177, Bio-Rad Laboratories, Inc, Hercules, CA, USA), and blocked with 5% nonfat milk (1706404, Bio-Red Laboratories, Inc, Hercules, Ca, USA), prepared 1X Tris-buffered saline with 0.5% Tween-20 (TBST). The membrane was incubated in primary antibodies overnight at 4°C in respective antibodies. Antibodies used are p-JNK/SPAK 1:1000 (4668, cell signaling technology, Danvers, MA, USA), and acrolein 1:1000 (ab48501, Abcam, Cambridge, MA USA). On the next

day, membranes were washed with TBST (three times, 5 min each) followed by incubation with respective HRP conjugated secondary antibodies (GE-Healthcare, Piscataway, NJ, United States). Membranes were washed with TBST (three times, 10 min each. followed by detection using an enhanced chemiluminescence system (GE-Healthcare, Piscataway, NJ, USA). Densitometry analysis was performed using ImageJ software and normalized to loading controls.

Statistical analysis

Statistical analysis was done using GraphPad Prism version 8 software (GraphPad, San Diego, CA, USA). Outliers were eliminated using Grubbs' outliers test at a significance level of 0.05. Two-way ANOVA followed by Tukey's multiple comparisons test or Student's t-test was used to analyze the significance level. A $p<0.05$ was considered statistically significant. Results are presented as Mean ± SEM.

Results:

Retinal vascular development is normal in SMOX Tg retina.

We first examined the impact of SMOX overexpression on retinal vascular development in newborn pups under normoxia conditions. Both WT-RA and SMOX Tg RA mice were euthanized at P17, and retinas were enucleated, stained with IB4, and flat-mounted. As presented in Figure 1, the images of retinal flat mounts presented a normal vascular development in both retinas (Figure 1A&B), outlining that neuronal SMOX overexpression has no deleterious effect on vascular growth in the retina under normal conditions.

Overexpression of neuronal SMOX aggravates OIR induced vaso-obliteration.

Vasoobliteration or capillary free zone around the optic nerve is a major hallmark of the OIR model [34]. Our previous studies have elucidated a remarkable expression of SMOX in OIR and diabetes mellitus models. Specifically, the expression was higher in retinal neuronal cells. Hence, we were interested to investigate the involvement of neuronal SMOX in vaso-obliteration [26, 28]. To study the impact of SMOX overexpression on OIR-induced vaso-obliteration, retinas from WT-OIR and SMOX Tg-OIR were isolated at P17, stained with IB4 to visualize the blood vessels, and analyzed to determine the vaso-obliterative areas. The images display that OIR-Induced vascular loss was greater in SMOX Tg-OIR retinas compared to WT-OIR (Figure 2A&B). Quantification of the avascular areas using ImageJ was shown to be significantly higher in SMOX Tg-OIR retinas, ($p < 0.05$). Figure 2C). These findings indicate that overexpression of neuronal SMOX exacerbates vaso-obliteration in the OIR retina.

Overexpression of neuronal SMOX increases OIR induced neovascularization.

Next, we sought to examine the effect of neuronal SMOX overexpression on retinal neovascularization, another pathological characteristic that accompanied OIR during the hypoxic

phase (P12-P17) [34]. Analysis of isolectin B4 staining of retinal flat-mounts at P17 illustrated a significant increase in neovascular tufts formation in SMOX Tg mice compared to WT mice (Figure 3A&B). This observation was confirmed by quantification of neovascularization using (SWIFT_NV step by step) depicting more tufts formation in SMOX Tg group compared to WT group ($p < 0.05$) Figure 3E) [33], demonstrating that overexpression of neuronal SMOX aggravates OIR-induced neovascularization.

Overexpression of neuronal SMOX slighty increases reactive gliosis.

Reactive gliosis is another hallmark of the OIR retina and is characterized by the high expression of GFAP [35]. Normally, GFAP is expressed by astrocytes, but in retinal injury, it is expressed by Muller cells [40]. In the present study, reactive gliosis was assessed using GFAP immunolabelling of retinal cryostat sections at P17. Immunolocalization analysis demonstrated increased GFAP expression in SMOX Tg-OIR retinas compared to WT-OIR retinas (Figure 4A-C). Our imaging studies clearly illustrate the upregulation of GFAP across the retinal layers including retinal ganglion cells (GCL), inner plexiform layer (IPL), and inner nuclear layer (INL). These findings demonstrate the increased injury and the involvement of neuronal SMOX in increasing glial activation.

MDL 72527 treatment does not affect room air (control) retinas.

MDL 72527 is a competitive inhibitor of spermine oxidase, [36]. In order to address the effect of SMOX inhibition on normoxic retinas, if any, MDL 72527 (40 mg/kg from P12 to P16) or vehicle (0.9% saline) was injected intraperitoneally to WT-RA mice. Analysis of retinal flat-mounts stained with isolectin B4 on P17 demonstrated normal vascular growth not only in vehicle-treated retinas, but in MDL 72527 treated ones as well (Figure 5A&B). Our findings suggest that MDL 72527 has no negative effect on retinal vasculature.

MDL 72527 treatment reduces OIR-induced vaso-obliteration.

It is well established that OIR treatment-induced vaso-obliteration [37]. Our previous study has illustrated that in comparison with vehicle treatment, administration of MDL 72527 from P6 to P12 reduced vascular injury during the hyperoxic phase in the OIR model [38]. Yet, the effect of this agent on the hypoxic phase is still unspecified. In the present study, we investigated the impact of MDL treatment on vaso-obliteration during the hypoxic phase. As shown in Figure 6, it is evident that OIR-induced vaso-obliterative areas are decreased in MDL treated mice compared to the vehicle-treated group (Figure 6A&B). Quantification of the results shows that MDL treatment significantly reduced OIR induced vascular loss compared to vehicle treatment, ($p < 0.05$) (Figure 6C). These results tie well with our previous studies wherein SMOX blockade lessened vascular injury in the hyperoxia phase of OIR [38].

SMOX inhibition reduces OIR-induced neovascularization.

We further assessed the effect of SMOX inhibition on neovascular tufts formation in the OIR model. The analysis of Isolectin B4 immunostaining displayed less neovascular tufts formation in MDL treated group compared to vehicle-treated retinas (Figure 7A&B). This was evident with Image J quantification and statistical analysis that neovascularization is significantly reduced with MDL treatment, ($p < 0.05$) (Figure 7C). This data advocates the hypothesis that SMOX activation is involved in OIR-induced pathological angiogenesis in the retina.

Acrolein conjugates are elevated in the OIR retina.

Next, we aimed to investigate the molecular pathways of oxidative damage and cellular stress signaling. Acrolein is a downstream effector of SMOX and polyamine oxidation and a known mediator of oxidative damage in diabetic retinopathy [39]. However, its involvement in the OIR

model has not been studied yet. Western blot analysis of P17 retinal lysates showed increased levels of acrolein conjugates in both OIR groups (vehicle and MDL 72527 treated) compared to RA control groups (Figure 8A). This finding demonstrates the implication of acrolein in OIR pathogenesis.

Stress signaling is altered in the OIR retina.

In addition, we looked at the p-JNK/SAPK signaling, the stress-activated protein kinase. Our results showed reduced levels of p-JNK/SAPK at postnatal day 17 (peak of pathological angiogenesis) in both OIR groups compared to RA control groups (Figure 9A). These studies need further investigation at the earlier time points of the ischemic phase.

Discussion

In the present study, we provide evidence for the first time that neuronal SMOX overexpression exacerbates the vascular injury in a model of ischemic retinopathy, and that treatment with MDL72527, an inhibitor SMOX limits the vascular injury. Retinal vascular injury in ischemic retinopathy is characterized by the failure of retinal vascularization, resulting in compensatory pathological angiogenesis to re-equilibrate with metabolic demands [40, 41]. However, the exact mechanism mediating this process is still elusive. Ischemic retinopathies are not merely vascular diseases, they are associated with neuronal dysfunction, gliosis, and inflammation as well [42-45].

Although the role of polyamine oxidation was partially defined in cancer and brain injury model [22, 23, 46, 47], its impact on retinal vascular injury is not clear. Utilizing the OIR mouse model, our laboratory has shown that hyperoxia-induced polyamine oxidation mediated retinal vascular degeneration through an elevation in spermine oxidase level, and its blockade with MDL 72527 limited retinal vascular injury. However, this study was purely focused on the hyperoxia phase

(P7-P12) [38]. In the present study, we report that neuronal SMOX overexpression does not affect normal retinal vascular development in normoxia, however, in hypoxia, it aggravates both retinal vaso-obliteration and vaso-proliferation. On the contrary, treatment with MDL72527 ameliorates the aforementioned pathological changes beyond its harmless effect on retinal vascular development. In addition, neuronal SMOX overexpression increased retinal astrogliosis. To the best of our knowledge, this is the first study to demonstrate the impact of neuronal SMOX on the retinal vascular injury in the hypoxia phase of the oxygen-induced retinopathy model.

Polyamines are vital for cell growth, differentiation, and proliferation [14, 15]. However, alterations in polyamine metabolism have been linked to various neurodegenerative diseases such as brain injury and cerebral ischemia [19, 46]. Our previous studies have shown the involvement of polyamine oxidation in both neuronal and vascular pathological changes in the OIR model. This was evident with the high level of SMOX in inner retinal neurons during the hyperoxia phase [26]. In our present study, we first examined the effect of neuronal SMOX overexpression on normal vascular development under normoxic condition. Staining of both P17 WT-RA and SMOX Tg-RA retinal flat-mounts with IB4 showed physiological retinal vascular growth in both retinas (figure3A-B). These findings outline that neuronal SMOX overexpression has no deleterious effect on vascular growth in the retina under normal condition.

During postnatal development, critical physiological changes occur in the retinal vasculature. These changes start with forming superficial vascular plexus originating from the optic nerve during the first week after birth, reaching retinal edges at P8. Then these superficial capillaries begin to sprout vertically to form deep and intermediate plexus, at P12 and P15, respectively. Lastly, these three vascular plexuses become fully mature by the end of the third postnatal week [34, 37]. Vasoobliteration or capillary free zone around the optic nerve and neovascularization are

major hallmarks of the OIR model [34]. Our previous studies have elucidated a remarkable expression of SMOX in OIR (P7-P12) and diabetes mellitus models [26, 28]. In the present study, analysis of retinal flat-mounts demonstrated significantly higher avascular areas and neovascular tufts in SMOX Tg-OIR retinas compared to the WT-OIR group. Together, these findings undoubtedly indicate that neuronal SMOX is involved in causing retinal vascular injury in OIR.

Glial cell activation and increase GFAP expressions are features of various retinal diseases including ROP, diabetic retinopathy, and ischemia-reperfusion injury [48-50]. These observations were confirmed in our laboratory in different retinal disease models [49, 51]. Consistent with these findings, our data showed that GFAP expression is higher in response to SMOX overexpression compared to WT OIR. It possible that the activation of glia is contributing to the oxidative stress generated as a result of polyamine oxidation [52].

MDL 72527 is an irreversible competitive inhibitor of SMOX and APAO [53]. In a rat model of cerebral ischemia, treatment with MDL72527 remarkably lowered brain edema, volume of ischemic injury, and polyamine levels [54]. Adopting the same animal model, inhibition of polyamine oxidation with MDL 72527 was shown to exert a neuroprotective effect after traumatic brain injury[55]. In addition, MDL72527 was illustrated to reduce oxidative stress in different cancer models [23, 56, 57]. We have previously shown that treatment with MDL 72527 reduced hyperoxia-induced retinal neurodegeneration, glial activation, and vascular injury [26, 38]. Further, our studies demonstrated that inhibition of SMOX with MDL72527 prevented neurodegeneration in models of retinal and diabetic retinopathy [27, 28]. In the present study, we demonstrated that MDL 72527 treatment during P12-P16 did not alter the normal development of retinal vasculature.

Retinal vascular injury owing to hyperoxia has been reported in ROP patients [58, 59] and animal models [59, 60]. Utilizing the OIR mouse model, our laboratory has previously elucidated that MDL 72527 treatment lowered vascular dropout and ameliorated vessels sprouting during hyperoxia [38]. Here, we further investigated the effect of MDL72527 in the hypoxic phase of OIR. Our results showed significantly reduced retinal avascular area and neovascular tufts in response to MDL 72427 treatment. Together, these findings confer that MDL 72527 is protective against OIR-induced retinal injury. This could be mediated by the ability of MDL 72527 to reduce oxidative stress. However, further studies are needed to unravel the mechanism behind it.

Acrolein is involved in the progression of various disease pathologies including cardiovascular disease, atherosclerosis, kidney disease, multiple sclerosis, and brain infarction [61-65]. Acrolein mediates its harmful effect by causing inflammation, DNA damage, ROS formation, protein adduction, endoplasmic reticulum stress, and mitochondrial dysfunction [25]. Acrolein is generated as a byproduct of polyamine oxidation [66]. Previously we have shown that level of conjugated acrolein was elevated in the diabetic retina, and treatment with MDL 72527 reduced the upregulation [28]. In the present study, we demonstrated for the first time the involvement of acrolein in the OIR model. High levels of acrolein conjugates are observed in WT-OIR retina treated with vehicle. However, treatment with MDL 72527 failed to reduce the conjugated acrolein levels in the OIR retina. Further studies are required at the earlier stage of ischemia to study the impact of MDL 72527 treatment in regulating acrolein levels in the OIR retina. JNK/SAPK plays an important role in cell survival, apoptosis, and proliferation [67]. Our laboratory has previously demonstrated that the maximal level of apoptosis in the OIR model during the ischemic phase was at P14 [49]. In the present study, we examined the effect of MDL 72527 at P17, the peak of pathological angiogenesis in WT-OIR retinas. Our results showed reduced levels of p-JNK/SAPK

in both OIR groups, compared to the control groups. Endothelial cells in the OIR retina are in the proliferative phase at P17 and that could be the reason for reduced p-JNK/SAPK observed in MDL72527 or vehicle-treated OIR retinas. The slight upregulation observed in the p-JNK/SAPK in MDL 72527 treated group shows the protective effect of SMOX blockade in pathological angiogenesis.

In conclusion, to the best of our knowledge, our study is the first report elucidating the impact of neuronal SMOX on vascular damage in a mouse model of ischemic retinopathy. In this study, we demonstrated that neuronal SMOX worsens retinal vascular injury through aggravating retinal vaso-obliteration, vaso-proliferation, and glial activation. In contrast, inhibiting SMOX function using MDL 72527 treatment was effective in limiting the pathological changes. Considering the urgent need to identify new therapeutic modalities for retinal neurovascular diseases, our data suggest the critical involvement of neurons in causing vascular damage, and that blocking neuronal SMOX signaling can be considered a potential therapeutic target to limit the progression of ischemic retinopathies.

Figure legends.

Figure. 1. Retinal vascular development is normal in SMOX Tg retina. A &B) Retinas from WT and SMOX Tg mice maintained at normoxia were isolated at P17, flat-mounted, and stained with isolectin B4. Images demonstrate normal retinal vascular development in both WT-RA and SMOX-RA. N= 3 in each group, P17-postnatal day 17, WT-RA- wild type room air, SMOX Tg-RA-spermine oxidase transgenic room air, IB4-isolectin B4, and representative images are shown. Scale bar =50 μm.

Figure. 2. Overexpression of neuronal SMOX aggravates OIR-induced vaso-obliteration. A&B) Retinal flat-mounts from WT-OIR and SMOX Tg-OIR stained with Isolectin B4 at P17, indicating the marked avascular area. C) Quantification of the avascular area using Image J shows significantly higher vaso-obliteration in SMOX Tg-OIR retinas compared to WT-OIR, (** $p < 0.01$; N=12 per group). Scale bar = 50 μm, and representative images are presented.

Figure. 3. Overexpression of neuronal SMOX increases OIR induced neovascularization. A&B) Images of Isolectin B4 staining of retinal flat-mounts from WT-OIR and SMOX Tg-OIR at P17. The formation of neovascular tufts is visualized in both groups. C&D) Representation of the neovascular tufts selected using Image J software, in the flat-mounts of OIR treated WT and SMOX Tg retinas. E)Image J quantification of neovascular tufts demonstrating a significant increase in SMOX Tg-OIR retinas compared to WT-OIR, (*$p < 0.05$; N = 12 per each group). Scale bar = 50 μm and representative images are presented.

Figure. 4. Overexpression of neuronal SMOX slightly increases glial activation. A-D) Representative confocal images of retinal sections from WT-RA, WT-OIR, SMOX Tg-RA, and

SMOX Tg-OIR, immunolabelled with GFAP, a marker of active gliosis. Increased gliosis was observed in both OIR groups. E) Quantification of GFAP expression in the OIR groups, demonstrating higher glial activation in SMOX Tg-OIR, however, the upregulation is not statistically significant. N = 3-5 per group, GCL;retinal ganglion cells, IPL;inner plexiform layer, INL; inner nuclear layer, ONL; outer nuclear layer, OPL; outer plexiform layer and representative images are presented.

Figure. 5. MDL 72527 treatment does not affect room air (control) retinas. A&B) Retinal flat-mounts isolated from WT-RA mice treated with vehicle or with MDL 72527, showing normal physiological growth of retinal vasculature. N = 3 per group and representative images are shown. Scale bar = 50 μm.

Figure. 6. MDL 72527 treatment reduces OIR-induced vaso-obliteration. A&B) Representative images of retinas isolated from WT-OIR mice treated with vehicle or MDL 72527. Blockade of SMOX reduced OIR-induced vaso-obliteration compared to vehicle-treated group. C) Quantification data showing a significant reduction in the avascular area in MDL 72527 treated WT-OIR retinas compared to the vehicle-treated group. (**$p < 0.01$; N = 5-6 per group). Scale bar = 50 μm and representative images are presented.

Figure. 7. MDL 72527 treatment reduces OIR-induced neovascularization. Figure. A&B) Representative images from retinal flat-mounts stained with Isolectin B4 showing that MDL 72527 treatment reduced OIR induced retinal neovascularization, compared to the vehicle-treated group. C&D) Representation of the neovascular tufts in the flat-mounts of WT-OIR retinas treated with Vehicle or MDL 72527. E) Image J Quantification data showing a significant reduction in tufts formation in the MDL 72527 treated WT-OIR retinas compared to the vehicle-treated group. (*$p < 0.05$); N = 5-6 per group; Scale bar = 50 μm and representative images are presented.

Figure. 8. Acrolein conjugates are elevated in OIR retinas. A) Western blot analysis of acrolein conjugates expression in P17 retinal lysates. B) Quantification of western blot showing increased levels of acrolein conjugates in WT-OIR retinas treated with vehicle or MDL 72527, compared to RA control groups. However, these changes are not statistically significant. N = 3 per group.

Figure. 9. Stress signaling is altered in the OIR retina. A) Western blot studies of p-JNK/SAPK expression, both phospho-54 and phosphor-46 in P17 retinal lysates. B) Quantification of phospho-54 JNK/SAPK showing reduced levels of phospho-54 JNK/SAPK in OIR retinas treated with vehicle or MDL 72527, compared to RA control groups. However this reduction was not statistically significant. C) Quanatification of phospho-46 JNK/SAPK showing significant higher expression of phospho-46 JNK/SAPK in WT-RA retinas treated with both vehicle and MDL 72527 compared to WT-OIR retinas treated with vehicle. (*$p < 0.05$, **$p < 0.01$). N = 3 per group.

Figures

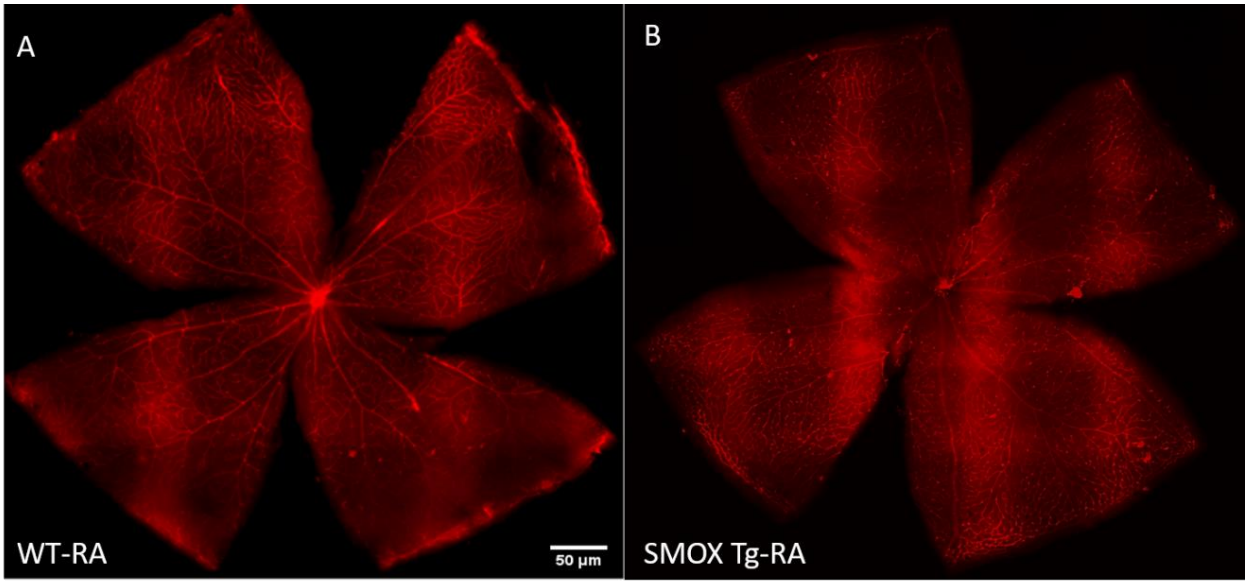


Figure. 1. Retinal vascular development is normal in SMOX Tg retina.

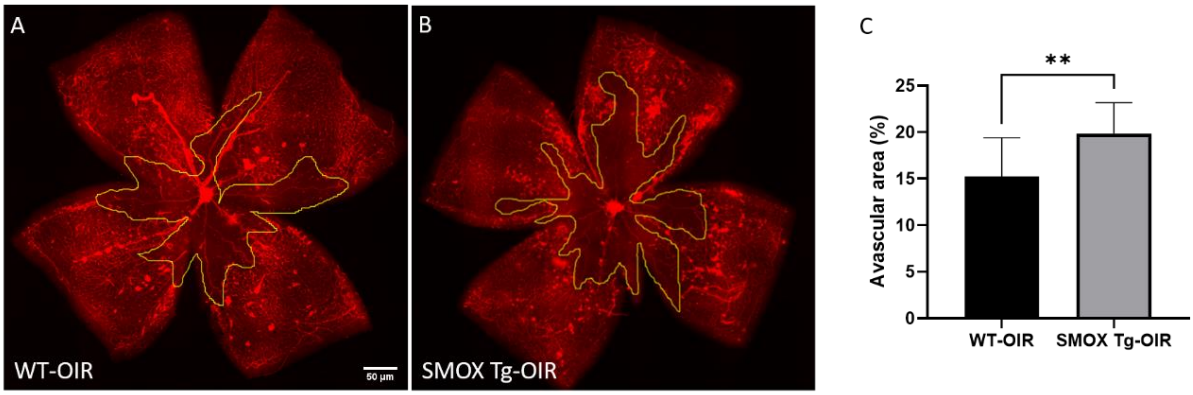


Figure. 2. Overexpression of neuronal SMOX aggravates OIR induced vaso-obliteration.

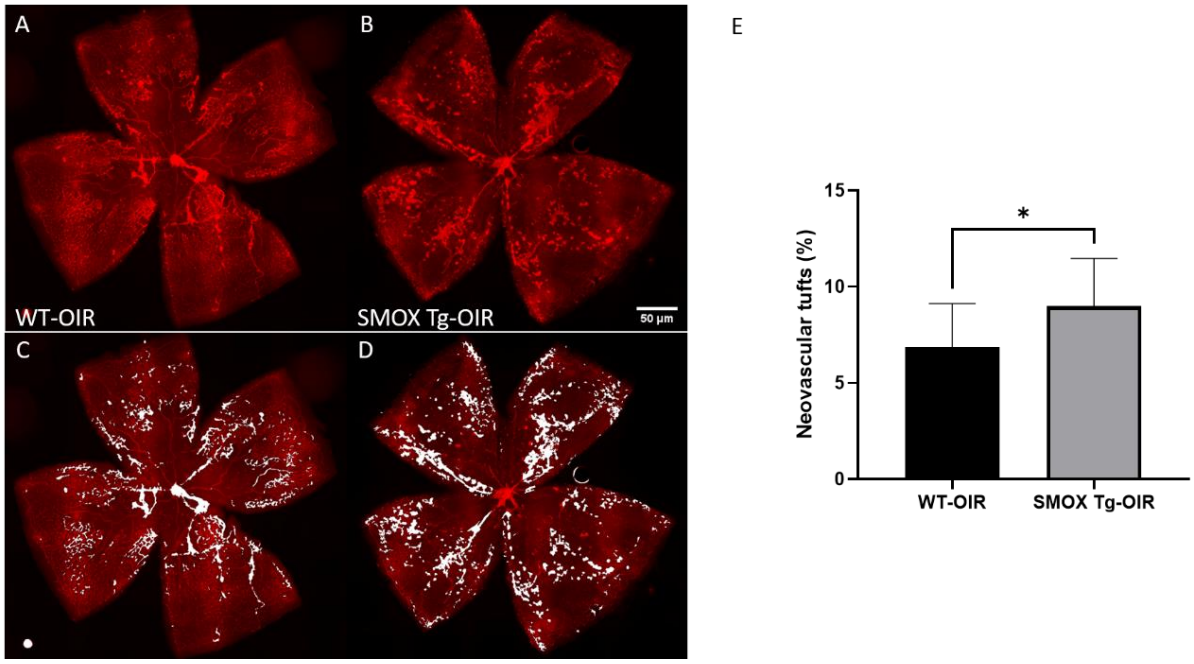


Figure. 3. Overexpression of neuronal SMOX increases OIR induced neovascularization.

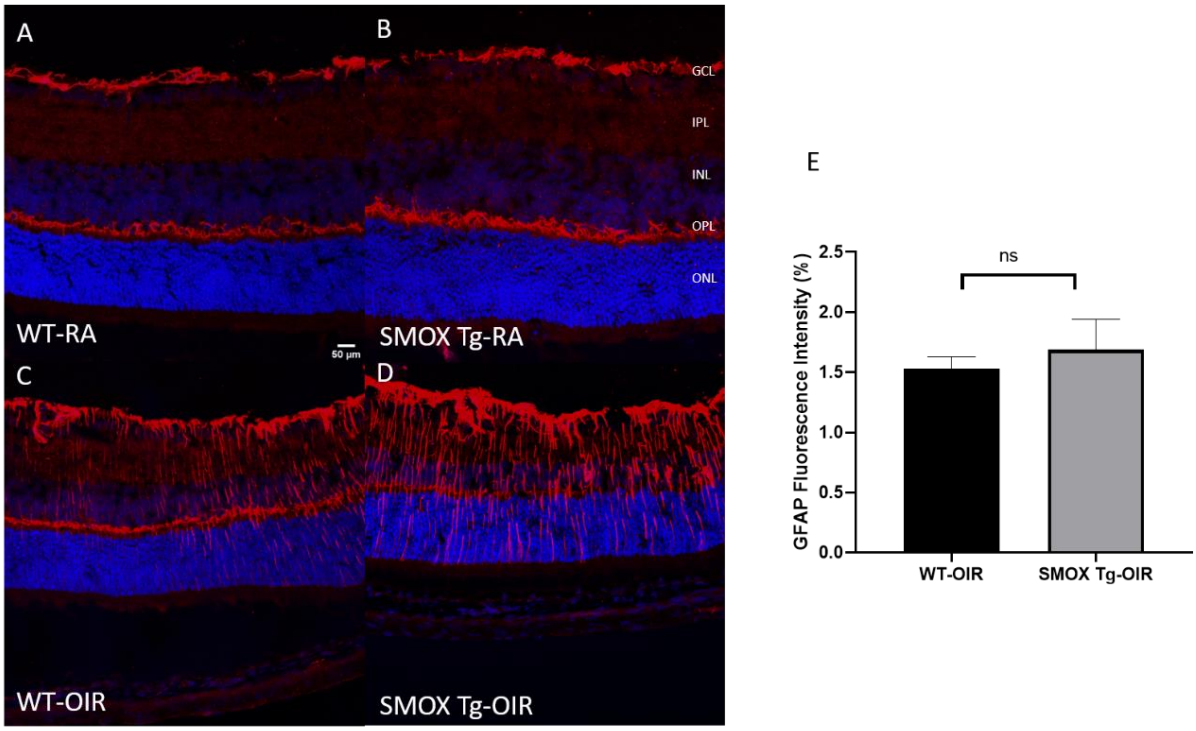


Figure. 4. Overexpression of neuronal SMOX slightly increases glial activation.

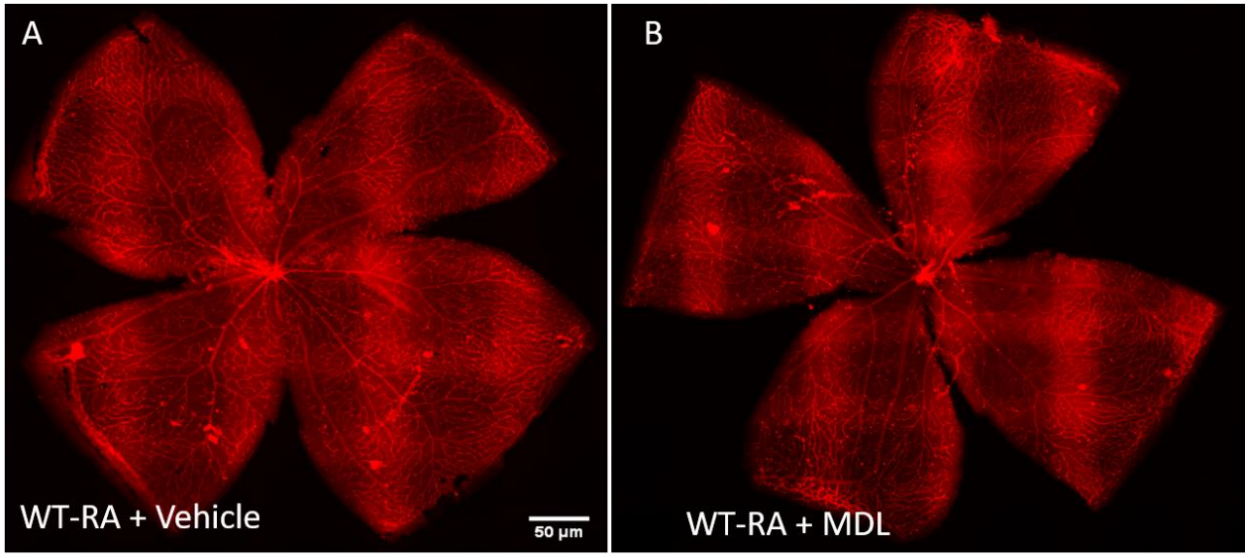


Figure. 5. MDL 72572 treatment does not affect room air (control) retinas.

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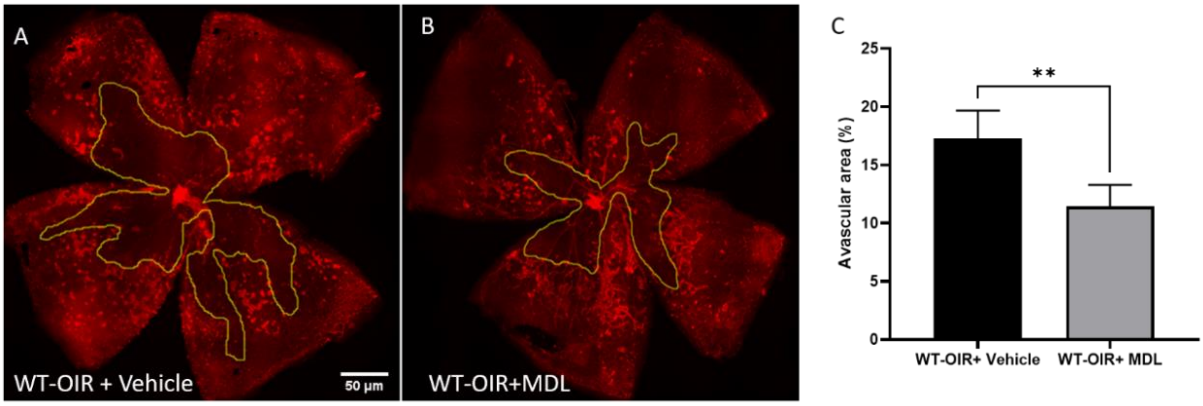

Figure. 6. MDL 72527 treatment reduces OIR-induced vaso-obliteration.

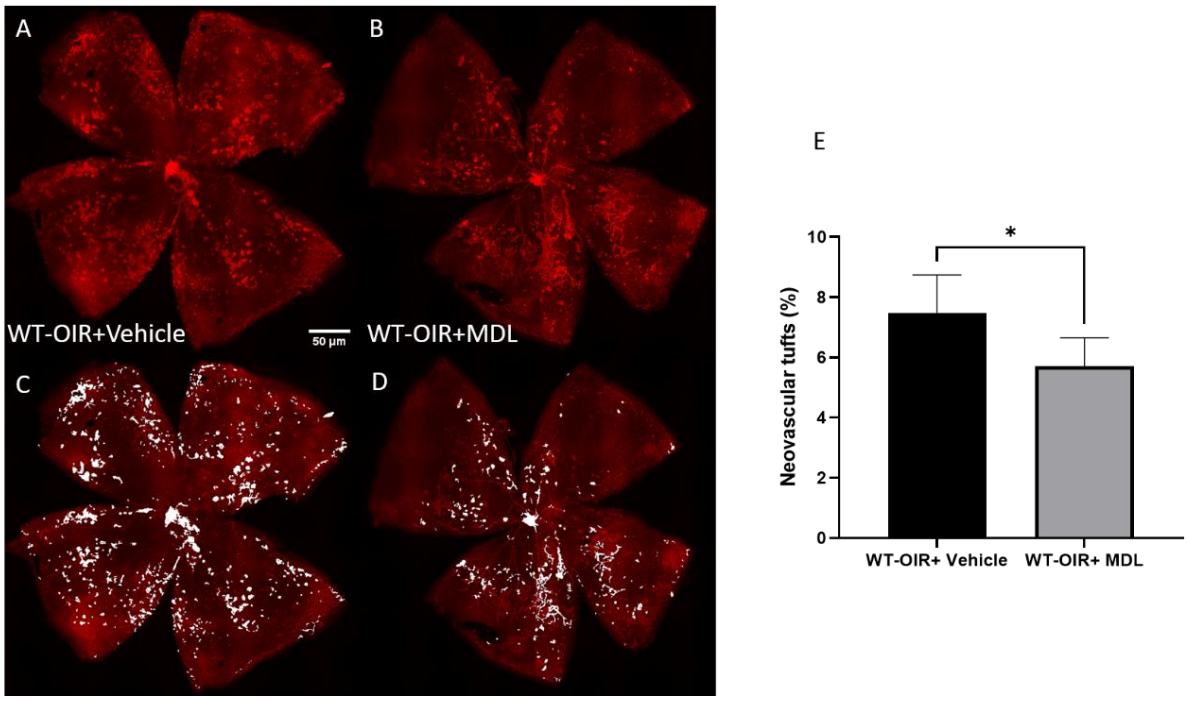

Figure. 7. MDL 72527 treatment reduces OIR-induced neovascularization.

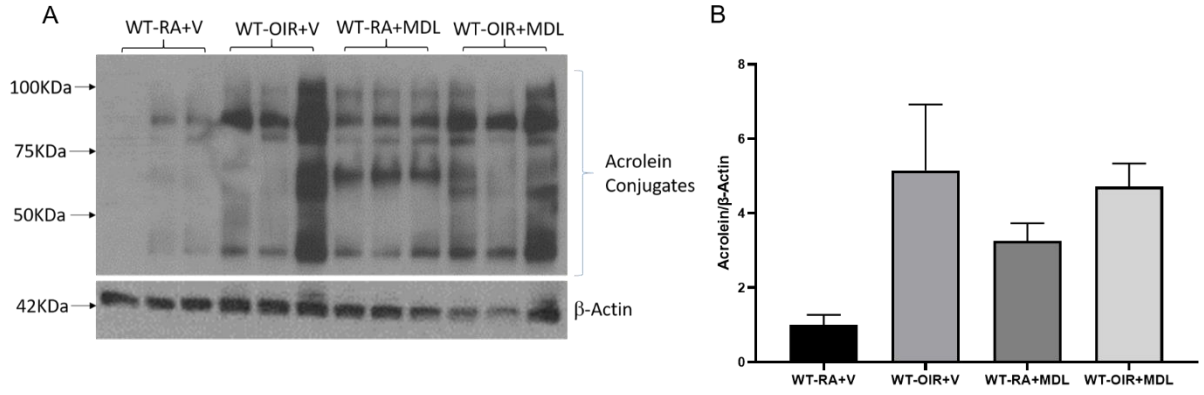

Figure. 8. Acrolein conjugates are elevated in OIR retinas.

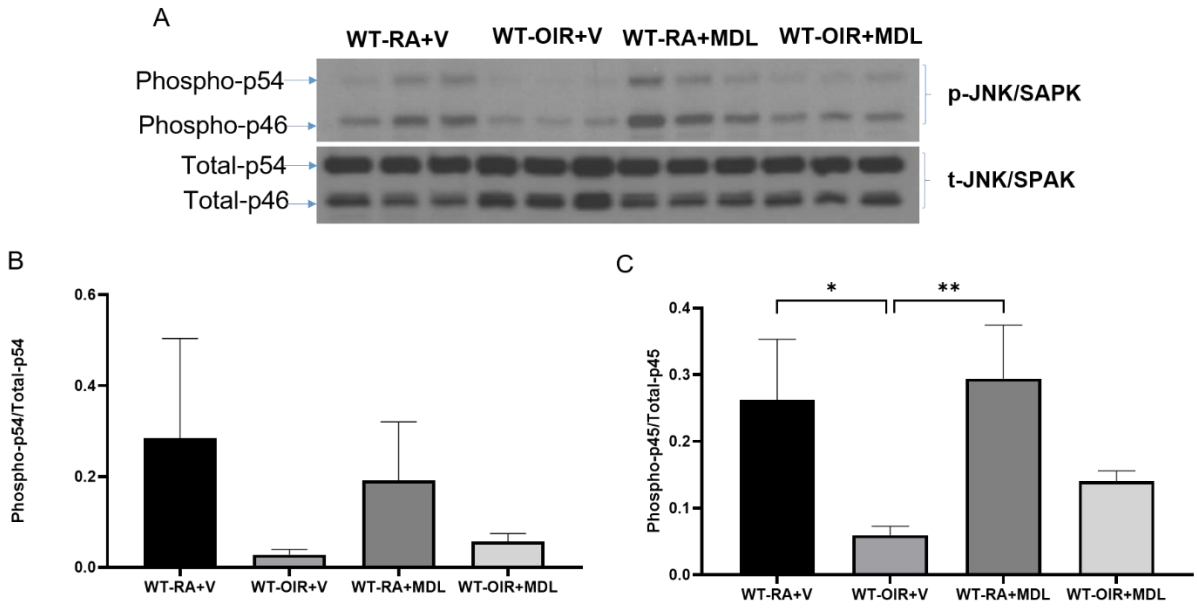

Figure. 9. Stress signaling is altered in the OIR retina.

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