An Eye Opener in Stroke: Mitochondrial Dysfunction and Stem Cell Repair in Stroke-induced Retinal Ischemia

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An Eye Opener in Stroke: Mitochondrial Dysfunction and Stem Cell Repair in Stroke-induced Retinal Ischemia

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Neuroscience
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List of Abbreviations

4,6-diamidino-2-phenylindole (DAPI)
Adenosine triphosphate (ATP)
Adenosine diphosphate (ADP)
Alzheimer’s disease (AD)
Apoptosis-inducing factor (AIF),
Apoptotic-protease-activating factor-1 (Apaf-1)
B-cell lymphoma-2 (Bcl-2)
Blood brain barrier (BBB)
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)
Central nervous system (CNS)
Central retinal artery occlusion (CRAO)
Common carotid artery (CCA),
Cortical spreading depolarization (CSD)
Damage-associated molecular patterns (DAMPs)
Deoxyribonucleic acid (DNA)
Dulbecco’s Modified Eagle Media/F-12 (DMEM)
Dulbecco’s phosphate-buffered saline (DPBS)
Dynamin-related protein 1 (Drp1),
Electron transport chain (ETC)
Extracellular vesicles (EVs)
Fetal bovine serum (FBS)
Glutathione peroxidase (GSHPx),
Glutathione-S-transferase (GSH).
High mobility group box-1 (HMGB1),
Inhibitor-of-apoptosis proteins (IAPs),
Intraocular pressure (IOP)
Mesenchymal stem cells (MSCs)
Middle cerebral artery (MCA).
Mitochondrial DNA (mtDNA)
Mitochondrial permeability transition pores (mPTPs)
N-methyl-D-aspartate (NMDA)
NADPH oxidase (NOX)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
Mitofusin 1 (Mfn1)
Mitofusin 2 (Mfn2)
Oxygen glucose deprivation (OGD)
Optic atrophy protein 1 (OPA1)
Oxidative phosphorylation (OXPHOS),
Oxygen consumption rate (OCR),
Parkinson’s disease (PD)
Paraformaldehyde (PFA)
Phosphate-buffered saline (PBS)
Reactive nitrogen species (RNS)
Reactive oxygen species (ROS)
Retinal pigmented epithelium (RPE)
Superoxide dismutase (SOD),
Tissue-type plasminogen activator (tPA),
Toll-like receptors (TLRs)
Tunneling nanotubes (TNTs)
Tumor necrosis-alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6)
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)
Abstract

Stoke is a leading cause of disability and mortality across the globe, making it a global health crisis. However, treatments for stroke remain limited with narrow therapeutic time window. Visual impairment negatively affects patients’ quality of life. During stroke, the disruption in blood flow might affect both brain and eye resulting in cerebral and retinal ischemia. Currently, there is a lack of treatment option that targets both cerebral and retinal ischemia. Ischemic stroke pathology is complex and multiphasic. The ischemic event is followed by a secondary cascade of inflammatory cytokines exacerbating the initial focal injury and expanding into the penumbra. In recent years, mitochondrial dysfunction has been identified as a hallmark pathological of ischemia and reperfusion injuries. This pathology is marked by an increase in oxidative stress and reduced oxidative phosphorylation. Investigations have begun to shine the spotlight on mitochondrial dysfunction as the key contributor in stroke pathology. The role of stem cells as a therapeutic option has been growing over the last decade, particularly in central nervous system disorders. However, the underlying therapeutic mechanisms of stem cell therapy remain elusive. In the present work, we investigated the role of mitochondrial dysfunction as a key mechanism of cell death in stroke-induce retinal ischemia and explored the therapeutic mechanism of stem cell to rescue the mitochondrial dysfunction. We demonstrated that retinal ischemia accompanied cerebral ischemia, causing cellular degeneration in the eye. We showed that mitochondrial dysfunction characterized by decreased mitochondrial function, altered mitochondrial network morphology, and imbalanced mitochondrial dynamics, altogether indicating that impaired mitochondria are a key contributor to ischemic cell death. Finally, we demonstrated that stem
cell therapy is a potential treatment for retinal ischemia by rescuing mitochondrial dysfunction via transfer of healthy mitochondria to injured cells. The present work revealed that using stem cells to target mitochondrial dysfunction represents a potential therapy for cerebral and retinal ischemia and potentially for other CNS disorders with mitochondrial dysfunction as a rampant underlying pathology.
Chapter 1: Introduction

Note to Readers: Portions of this chapter have been previously published in “Nguyen H, Liska MG, Crowley MG, Borlongan CV. Stem Cell-Paved Biobridge: A Merger of Exogenous and Endogenous Stem Cells Toward Regenerative Medicine in Stroke. In: Lapchak PA, Zhang JH, editors. Cellular and Molecular Approaches to Regeneration and Repair. Cham: Springer International Publishing; 2018. p. 153-80.”, and has been included with permission from the publisher.

1.1. Introduction to Stroke and Current Treatment Strategies

Stroke is defined as a pathological state whereby a reduction in blood flow affects one or more regions of the brain, which may be caused by an obstructed vessel resulting in ischemic stroke or a ruptured blood vessel leading to hemorrhagic stroke. While ischemic stroke is more common, hemorrhagic stroke has a higher mortality rate. Stroke is one of the leading causes of death and disability in the United States with approximately 800,000 people affected annually. Stroke survivors experience a range of long-term consequences from moderate functional impairments to severe disability. The estimated economic burden of stroke is $34.3 billion annually including the healthcare cost and loss of productivity, with the projected figure to increase to $94.3 billion in 2035. In fact, the economic burden of stroke has increased due to improved treatment protocols, resulting in the overall decreased in mortality rate. Despite the prevalence and severity of the economic burden, the therapeutic options for stroke are limited to tissue-type plasminogen activator (tPA), endovascular interventions, and physical therapies to
alleviate symptoms. Only a small percentage of stroke patients are administered tPA primarily because of ischemic stroke-related eligibility criteria and its narrow 4.5 hours from onset therapeutic window, with the risk of hemorrhagic transformation increasing outside the recommended timeframe. Similarly, not all patients are qualified for mechanical thrombectomy due to many factors such as the location of the embolus, time since onset, the collateral circulation, among others. In addition, endovascular interventions share the same risk of time-dependent hemorrhagic transformation. Based on the current state of limited stroke treatments, patients are left with physical abnormalities, including visual deficits, manifesting as debilitating symptoms. Therefore, there is a need for innovative and more effective therapies for both acute and chronic stage of stroke.

1.2. Visual Impairment in Stroke

Visual impairment is one of the factors that limits the likelihood of stroke patients participating in rehabilitation, displaying functional recovery, and exhibiting an increase quality of life. According to a prospective multicenter cohort study, visual impairments occurs in 92% of stroke patients. In another study, 20.5% of stroke patients display persistence visual impairment at 90 days. Furthermore, patients with monocular vision loss exhibit a higher risk of concurrent ischemic stroke and vice versa. Retinal ischemia is the major cause of visual impairment in approximately 16% of the stroke patients; and it is a shared pathology with other common ocular vascular diseases, such as diabetic retinopathy, glaucoma, retinal vein occlusion, and central retinal artery occlusion (CRAO). In particular, CRAO shares similar atherosclerosis risk factors with cerebral ischemia. Despite many pathological similarities between retinal ischemia and cerebral ischemia, the underlying mechanisms and the relationship
between them remain unclear which lead to a lack of effective treatment for retinal ischemia and stroke as a whole 21, 29.

1.3. Stroke Pathology

In both cerebral ischemia and retinal ischemia, ischemic stroke occurs when there is an occlusion of an artery, resulting in loss of blood supply to one or more regions. This causes a reduction in both the oxygen and nutrient supplies. The brain and the eye are one of the most metabolically active tissues and consume oxygen more than many other tissues 30. Due to the high demand of oxygen in the brain and eye, the oxygen availability in these tissues is limited by the blood flow and the consumption rate 30, 31. Even within the retina, the oxygen tension varies greatly depending on the retinal layer 32, 33. For example, oxygen tension is much higher in the choroid plexus and lower in photoreceptor and the ganglion cell layers 32, 33. Therefore, these tissues are highly sensitive to changes in oxygen level such as during hypoxic environment 34, 35. The nervous system and the retina require a high demand of energy to maintain membrane potential, trigger action potential, and communicate via chemical synapses 36, 37. Hence, neurons are sensitive to metabolic stress and susceptible to ischemic insult 38. The lack of energy supply causes ionic imbalance and mitochondrial damage, increases reactive oxygen species (ROS) production, and decreases membrane integrity which singly or in tandem contribute to cell death cascades 39, 40. The cell death after ischemic injury occurs in stages (acute, sub-acute, and chronic) starting within minutes and extending to hours, even days, weeks and months 41. The ischemic core, the initial irreversible damaged tissue, is less amenable to therapeutic treatment 42. The penumbra is the area surrounding the ischemic core in which the cells are under-distress due to low level of oxygen and nutrients and high level of toxic microenvironment spilled from the ischemic core 42, 43. The penumbra is an endangered region and could expand into an irreversible
injury if left untreated. Yet, the penumbra can potentially be rescued and has a wider therapeutic window making it the ideal target for interventional therapies.

1.3.1. Excitotoxicity

Within minutes of an ischemic attack, cells in the ischemic core fail to maintain ionic homeostasis, leading to acidosis, cell swelling and membrane rupture. The necrotic cells within the infarct core produce a toxic microenvironment. Leaked deleterious substances from these cells have the capacity to reach adjacent healthy cells and cause harm. Ruptured cells and failure of reuptake mechanisms contribute to high levels of glutamate in the microenvironment that lead to excitotoxicity in neighboring cells. Excess glutamate prolongs the stimulation of N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, further increasing influx of Na⁺, Ca²⁺ and water into the cells. The influx of Na⁺ and water contributes to cell swelling and edema. Activation of glutamate receptors does not account fully for the increase of intracellular Ca²⁺. In fact, Ca²⁺ can build up the cells through other channels such as Na⁺/Ca²⁺ exchanger, acid sensing ion channels and TRP channels. In addition, Ca²⁺ can be released from organelles such as mitochondria and the endoplasmic reticulum. The excessive calcium influx triggers the activation of proteases, nucleases and lipases which degrade proteins and essential cellular structures. Furthermore, the high level of glutamate and K⁺ triggers cortical spreading depolarization (CSD), a phenomenon in which a wave of depolarization propagates along neurons and astrocytes. In fact, CSD has been demonstrated strongly in stroke patients. Since repolarization is a high energy demanding process, CSD exacerbates the ionic imbalance and hastens the cell death process of cells with limited blood
supply in the penumbra $^{63,64}$. As a result, CSD contributes to the expansion of the ischemic infarct core $^{63}$.

1.3.2. Oxidative and Nitrative Stress

After the initial insult, there is a surge of ROS and reactive nitrogen species (RNS) $^{65,66}$. The oxidative and nitrative stress play a key role in the progression of ischemic injury $^{67,68}$. While a low balanced level of radicals is important for normal function of neuronal cells; over-production of ROS and RNS after ischemic insult exacerbates cellular damage $^{67,69}$. Excessive ROS and RNS overwhelm the antioxidant enzymes and damage macromolecules such as proteins, lipids and nucleotides $^{70-72}$. In addition, ROS and RNS also initiate cellular apoptosis via release of pro-apoptosis factors from mitochondria (further discussion in 1.5) $^{73}$. Beside mitochondria, other enzymes also contribute to the production of ROS, such as NADPH oxidase (NOX) and nitric oxide synthase (NOS) $^{74-76}$. In fact, NOX is implicated as a potent source of ROS. In fact, ROS from NADPH oxidase kindles further ROS production from mitochondria $^{75}$. Similar to ROS, RNS also has deleterious cellular effects, such as inhibiting mitochondrial enzymes, facilitating mitochondrial permeability transition pores (mPTPs) formation, causing deoxyribonucleic acid (DNA) strain breaks, activating PARP and increasing influx of Ca$^{2+}$ $^{77}$. In particular, nitric oxide (NO) covalently bind to cysteine residues forming S-nitrosothiol derivatives and altering protein functions $^{78-80}$. Taken together, ROS and RNS play an important role in the stroke pathology.

1.3.3. Neuroinflammation

As we mentioned above, the resulting pathophysiology of stroke extends into chronic phase, days and weeks after the initial insult. Neuroinflammation and the disruption of the blood
brain barrier (BBB) contribute significantly to the secondary cell death process during the chronic phase of stroke. After stroke, neuroinflammation has a bi-phasic response with double-edged sword effects. In the acute phase, the activation of microglia and astrocytes has neuroprotective roles such as removing dead cells, clearing debris, and facilitating tissue repair. However, prolonged inflammation perpetuates secondary cell death in chronic phase. Neuroinflammation is instigated by damage-associated molecular patterns (DAMPs) which are released from dead and dying cells. Examples of DAMPs are high mobility group box-1 (HMGB1), heat shock proteins, lipopeptides, advanced glycation end-products and hyaluronan. DAMPs activate their receptors such as toll-like receptors (TLRs) which lead to production of pro-inflammatory cytokines and chemokines via NF-κB signaling pathway. In particular, TLR2 and TLR4 expression have been shown to be upregulated following ischemia and correlate with infarct volume. Once the process is initiated, pro-inflammatory cytokines such as tumor necrosis-alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6) and others are released from various cell types leading to leukocytes infiltration and cell death. Studies have demonstrated that TNF-α and IL-1β promote neutrophil infiltration and exacerbate ischemic injury. For example, TNF-α and IL-1β increase the expression of adhesion molecules (ICAM-1, P-selectin and E-selectin) in endothelial cells and promotes rolling and sticking of leukocytes, which are critical to neuroinflammation-mediated tissue injury. Whereas TNF-α antibody and TNF-α binding protein have been shown to decrease infarct volume, IL-6 exhibit dual roles of pro-inflammation and beneficial for tissue repair. High level of IL-6 has been shown to elevate body temperature and associate with larger infarct size after stroke. Yet, IL-6 also inhibits TNF-α and promotes tissue remodeling via activation of STAT (signal transducer and activator of transcription). Furthermore, this stroke-induced inflammatory
response further exacerbates cell death and BBB breakdown\textsuperscript{84,112}. Rampant neuroinflammation disrupt cell-cell interactions of the neurovascular unit\textsuperscript{84}. Pro-inflammatory cytokines increase BBB leakage by altering tight junctions between endothelial cells, pericytes and astrocytes\textsuperscript{103}. The damaged BBB allows substances and cells which are normally foreign to the brain to enter and exacerbate the inflammation response. Finally, as mentioned above, inflammatory cytokines and chemokines stimulate the expressions of adhesion molecules which facilitate the infiltration of peripheral immune cells to enter the brain. Altogether, unregulated prolonged inflammation worsens the hostile ischemic environment and facilitates secondary cell death in the later stage of stroke.

1.4. Introduction to Mitochondria

Mitochondria are intracellular organelles enclosed by double membrane, creating two distinct compartments (the intermembrane space and the matrix)\textsuperscript{113}. The outer membrane has high permeability, allowing the free movement of ions, water, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and other small molecules into the intermembrane space\textsuperscript{113}. On the other hand, the inner membrane has more restricted permeability compared to the outer membrane\textsuperscript{113}. The inner membrane is the center of mitochondrial energy production, containing the electron transport chain (ETC) enzymes, ATP/ADP transport proteins, and other ion channels\textsuperscript{73}. Oxidative phosphorylation (OXPHOS), through ETC, produces the majority of energy in the form of ATP\textsuperscript{73}. The ETC comprises of five multi-enzyme complexes (complex I-V) which can also form supercomplexes in the inner membrane of the mitochondria\textsuperscript{114}. The electrons carried by NADH and FADH\textsubscript{2} are fed into the ETC at complex I and II to initiate the process\textsuperscript{114}. Complex I, III, and IV use the electrons to pump protons from the mitochondrial matrix into the intermembrane space, creating the electrochemical gradient for ATP production\textsuperscript{73,115}. Soluble
cytochrome c facilitates the transfer of electrons from complexes III to complex IV, the final electron acceptor. The proton motive force drives the ATP synthesis of complex V. Mitochondria not only produce energy in the form of ATP but also participate in maintaining homeostasis of the cells, regulating metabolism, apoptosis, and modulation of inflammation. Hence, mitochondrial dysfunction has been implicated as a key contributor to disease progress in stroke and other neurological disorders. In fact, mitochondrial dysfunction has been studied in stroke and in various neurological diseases e.g. fragile x-associated tremor/ataxia syndrome, Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease, and retinal ischemia and optic neuropathy.

1.5. Mitochondrial Dysfunction in Stroke

The central nervous system (CNS) consumes approximately 20% of the body’s total energy production. The majority of the generated energy is used to maintain the membrane potential, initiate action potential and communicate with other cells via chemical synapses. With the sudden loss of oxygen and energy supply in cerebral ischemia/retinal ischemia, mitochondria, the powerhouse of the cells, cannot maintain sufficient ATP production. As previously discussed, the lack of energy leads to disruption in membrane integrity, ionic imbalance and necrosis. Moreover, this failure of energy supply triggers a cascade of events lead to cell death and places mitochondria in the center of cerebral and retinal ischemia pathology. Therefore, dysfunctional mitochondria with altered bioenergetics will have detrimental effects to the cells and the CNS.

Under normal condition, small amount of ROS is produced during OXPHOS. The leakage of electrons at the sites, predominantly complex I and III, leads to the production of ROS such as superoxide, hydrogen peroxide, hydroxyl radical etc. As previously mentioned,
low level of ROS is important for cellular function and cells have developed antioxidant
enzymes to maintain the redox homeostasis such as superoxide dismutase (SOD), glutathione
peroxidase (GSHPx), glutathione reductase, and glutathione-S-transferase (GSH)\textsuperscript{138, 139}. Under
hypoxic environment such as ischemic stroke, mitochondria produce high amount of ROS that
overwhelm the antioxidant defense and damages macromolecules such as proteins, lipids, and
nucleotides\textsuperscript{73, 140}.

Mitochondria also play a major role in initiating apoptosis pathways\textsuperscript{73, 141}. Damaged
mitochondria caused by energy failure, intracellular Ca\textsuperscript{2+} overload, membrane depolarization,
and ROS can initiate the apoptosis process through the release of cytochrome c, apoptosis-
inducing factor (AIF), and Smac/DIABLO into cytosol via mPTPs\textsuperscript{142}. Accumulation of Ca\textsuperscript{2+}
cleaves B-cell lymphoma-2 (Bcl-2) interaction domain which in turn interacts with pro-apoptotic
proteins to open mPTPs. A transient opening of mPTPs further releases pro-apoptotic substances
(e.g. cytochrome c and AIF) into the cytosol and collapse the mitochondrial membrane potential.
Cytosolic cytochrome c interacts with apoptotic-protease-activating factor-1 (Apaf-1) to activate
pro-caspase-9, which in turn activates caspase-3\textsuperscript{143, 144}. Activation of caspase-3 leads to
mitochondrial membrane depolarization, DNA fragmentation and chromatin condensation.
Conversely, Smac/DIABLO activates caspases by inhibiting inhibitor-of-apoptosis proteins
(IAPs), which inhibit pro-caspase activation under normal condition\textsuperscript{145, 146}. Upon translocation
from the mitochondrial outer membrane to the nucleus, AIF accelerates apoptotic process
through DNA fragmentation and chromatin condensation\textsuperscript{147, 148}. Bcl-2 protein family is another
pathway that regulates apoptosis. Bcl-2 proteins can be classified into pro-apoptotic proteins
such as Bax, Bid, Bad etc. or anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Bcl-w\textsuperscript{73, 149, 150}. 

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Mitochondria are not a static organelles. In fact, mitochondria constantly undergo fission and fusion and alter the morphology of the mitochondrial network \textsuperscript{151-153}. Mitochondrial fission is the process in which mitochondria constrict and divide whereas mitochondrial fusion is merging of mitochondria. Dynamin-related protein 1 (Drp1), an important regulator of mitochondrial fission, is recruited to the outer membrane of mitochondria \textsuperscript{154, 155}. In combination with other proteins such as dynamin 2, Drp1 form a ring-like structure prior to splitting mitochondria \textsuperscript{156, 157}. On the other hand, there are several crucial proteins that participate in the process of mitochondria fusion such as optic atrophy protein 1 (OPA1) and mitofusin 1 and 2 (Mfn1 and Mfn2). Mitochondrial fission and fusion dynamics are essential for maintaining homeostasis of mitochondria. Mitochondrial fission facilitates the clearance of damaged proteins and mitochondrial DNA (mtDNA) via mitophagy \textsuperscript{158}. Moreover, in neuronal cells, mitochondrial fission is essential for transportation of mitochondria to long distance destination such as axon terminals and dendrites \textsuperscript{159}. In contrast, mitochondrial fusion enhances the mitochondrial functions by sharing the metabolites across the network, exchanging mtDNA, and allowing the production of ATP more efficiently \textsuperscript{160, 161}. Studies have demonstrated that ischemic injury alters mitochondrial dynamics characterized by an upregulation of Drp1, resulting in an increased mitochondria fission \textsuperscript{162}. Accordingly, mitochondria become fragmented and dysfunctional in ischemic cells \textsuperscript{162, 163}. Studies have shown that decreased Drp1 expression reduces mitochondrial ROS production and limits infarct volume \textsuperscript{164, 165}. Conversely, Mfn2 expression has been reported to be decreased in ischemic cells, while inhibition of Mfn2 and OPA1 further exacerbates cell death \textsuperscript{166}. Therefore, maintaining the balance between mitochondrial fission and fusion is critical to the survival of cells especially under pathological condition such as stroke.
Mitochondria also play an important role in modulating inflammation. Damaged fragments of mtDNA are released into the cytosol and act as DAMP, triggering inflammation. In particular, fragmented mtDNA activates TLR9, triggering NF-κB inflammation signaling pathway. In addition, mtDNA also leads to the activation of NLRP3 inflammasome which promotes the cleavage of pro-IL-1β to pro-inflammation IL-1β. Indeed, studies have demonstrated that NLRP3 protein level is increased together with elevated IL-1β and IL-18 level after ischemic stroke. Conversely, studies have shown that NLRP3-deficient mice or suppressing NLRP3 with immunoglobulin reduces infarct size and behavioral deficits.

In particular, mitochondria’s role as the powerhouse of the cellular machinery serves as the crux of maintaining homeostasis and its dysfunction plays a key role in cerebral and retinal ischemia pathology. Understanding the contribution of mitochondrial dysfunction in retinal ischemia pathology may provide mechanistic and translational insights into developing more effective treatments for stroke and other disorders with retinal ischemia pathology. Therapeutic strategies such as stem cell therapy that reverse mitochondrial dysfunction could be valuable options for retinal ischemia and other neurological disorders with mitochondrial pathology.

1.6. Introduction to Stem Cells

Stem cells are a class of cells which share specific characteristics such as self-replication, differentiation, and expression of specific cell markers. Stem cells can preserve their characteristics via self-replicating and can be found within several niches of the body. The differentiation capability of stem cells allow them to regenerate and maintain the homeostasis of the body. For example, bone marrow stem cells rejuvenate the red blood cells population in the body. Stemness is the term coined to describe the capacity of stem cells to self-replicate and
differentiate into various lineages\textsuperscript{181}. Stem cells are often classified based on their origin of harvest and their potency (e.g. totipotent, pluripotent, or multipotent)\textsuperscript{182}. For example, adult adipose multipotent stem cells are found in fat tissue and can differentiate into limited number of cell types in the same lineage. In addition, stem cells can also be classified further based on expressed cell markers. For example, Bone marrow-derived mesenchymal stem cells are positive for CD29, CD44, CD105, CD73, CD90, CD106, and CD166 markers, while negative for CD14, CD34 and CD45 \textsuperscript{183, 184}.

1.6.1. Stem Cells for Neurological Disorders

Since their discovery, stem cells have been studied as a potential therapeutic treatment for many neurological disorders such as stroke, traumatic brain injury (TBI), amyotrophic lateral sclerosis, AD, and PD\textsuperscript{185-191} and ocular degenerative diseases such as glaucoma, age-related macular degeneration, retinitis pigmentosa, and diabetic retinopathy\textsuperscript{192-199}. There are some ongoing limited clinical trials using stem cells therapy for neurological disorders, including stroke\textsuperscript{200, 201}. Despite early belief, stem cells exist in adulthood and contribute to the homeostasis of the body\textsuperscript{202-204}. To date, the well-established mechanism of action of stem cells are cell replacement\textsuperscript{205-208} and bystander effects (secretion of neuroprotective, neuroregenerative, anti-inflammatory molecules, and others)\textsuperscript{209-216}. The initial concept is that transplanted stem cells could replicate and differentiate to replace dead/dying cells. However, studies have shown that this notion of cell replacement may not be the optimal regenerative mechanism of stem cells\textsuperscript{217, 218}. Only small amount of transplanted stem cells survive despite best effort to control for immunogenicity through autologous transplant or using immunosuppressant drugs\textsuperscript{219}. To further discredit the cell replacement mechanism, even smaller amount of transplanted cells can differentiate into neuronal cells \textit{in vivo} models\textsuperscript{207, 208}. One possible explanation for the
phenomenon is that the hostile microenvironment of the disease brain does not support the survival, differentiation and maturation of stem cells. Moreover, the observation of neural network repair and functional integration of transplanted cells with the host cells remain elusive. Therefore, cell replacement may not be a major contributor to observed functional benefits of cell transplantation. Instead, accumulating studies support the bystander effects of stem cells in which secreted trophic factors and cytokines are the major contributors to the therapeutic mechanism of stem cells. For example, in animal studies, bone marrow-derived mesenchymal stem cells secrete a variety of trophic factors (e.g. vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, insulin growth factor-1, and hepatocyte growth factor) which promote the neuroregeneration process. In addition to growth factors, stem cells secrete microvesicles and exosomes containing anti-inflammatory cytokines to modulate inflammation. In fact, the current consensus has shifted from cell replacement paradigm toward bystander effect. However, both mentioned mechanisms cannot explain whole spectrum of observed benefits. For example, both mechanisms do not fully account for the endogenous tissue recovery effect observed after transplantation. To this end, recent studies from our laboratory have proposed a third mechanism in which exogenous stem cells facilitate the migration of endogenous stem cells from neurogenic niches to injured area. Moreover, the effect of stem cells on mitochondrial dysfunction remains to be fully investigated.

1.6.2. Targeting Mitochondrial Dysfunction using Stem Cells

Recent studies have shown that using stem cells to treat diseases characterized by mitochondrial dysfunction is a novel and promising approach for stroke. In particular, the concept of mitochondrial transfer between mammalian cells is intriguing and has been reported by many studies summarized in a recent review. However, the underlying mechanism remains
unclear and many early studies focus in non-nervous systems\textsuperscript{227-231}. Only recently, a study has demonstrated that astrocytes transfer mitochondria to neurons after stroke in both \textit{in vitro} and \textit{in vivo}\textsuperscript{232}. The stimulated astrocytes, via CD38/cyclic ADP ribose signaling, secrete extracellular mitochondria which provide therapeutic benefits for ischemic neurons\textsuperscript{232}. Currently proposed mechanisms for the mitochondrial transfer involve tunneling nanotubes (TNTs), extracellular vesicles (EVs), gap junctions and cell fusion\textsuperscript{228, 233-235}. TNTs are thin and long structures comprised of actin and microtubules that allow two separated cells to connect with each other\textsuperscript{235, 236}. On this note, Miro1, a Rho GTPases, plays a significant role in regulating the transfer of mitochondria through TNTs\textsuperscript{227, 229}. Bone marrow-derived stromal stem cells form connexin-43 containing gap junction with alveolar epithelial cells\textsuperscript{228}. This gap junction facilitates the transfer of mitochondria and increases ATP production in alveolar cells after LPS insult\textsuperscript{189, 228}. Cell fusion is another way in which stem cells can transfer mitochondria to recipient cells. Human MSCs have shown to fuse with injured respiratory tract epithelial cells\textsuperscript{233}. In a separate study, bone marrow-derived cells are shown to fuse with cardiomyocytes, hepatocytes and Purkinje neurons\textsuperscript{237}. Recent studies have shown that mitochondria can be transported via EVs, yet the detailed processes associated with such uptake with EVs remains to be investigated\textsuperscript{122, 238}. Of note, such transfer of mitochondria from stem cells to retinal cells and its mechanisms correspond to the main objectives of the present study on stroke-induced retinal ischemia.

1.7. \textbf{Summary and Hypothesis}

Stroke remains to be one of the leading causes of death and disability in America and contributes a significant social and economic burden to the society\textsuperscript{1}. Approximately, 16\% of stroke patients suffer retinal ischemia but it is often overlooked and under-reported\textsuperscript{14}. Visual impairments negatively affect functional ability, the likelihood of participation in rehabilitation,
and the quality of life of stroke patients. The underlying mechanisms of retinal ischemia and their relation with cerebral ischemia are not fully understood, resulting in limited holistic therapeutic treatment for stroke patients. Mitochondria play crucial roles in the cell homeostasis and function, such as energy production, regulating metabolism, apoptosis, cell cycle, calcium homeostasis, generation of ROS, and modulating inflammation. Mitochondrial dysfunction has been linked to multiple neurological diseases including stroke, AD, PD, and TBI. However, the role of mitochondrial dysfunction in retinal ischemia remains to be investigated. For the past few decades, stem cell therapy has emerged as a promising therapeutic approach to treat various neurological disorders. In particular, multiple pre-clinical studies have demonstrated the benefits of stem cell therapy for ischemic stroke. In addition, limited clinical trials of stem cell therapy for stroke have commenced over the last five years. Hence, stem cell therapy might pose as a potential therapeutic approach for retinal ischemia, which often accompanies cerebral ischemia. Despite decades of research, the mechanism(s) of stem cell therapy is not fully elucidated and remains as a fundamental gap in our knowledge. An elucidation of stem cell mechanism of action may improve the safety and efficacy, and advance the clinical application of stem cell therapy. Understanding the role of mitochondria dysfunction in retinal ischemia may provide insights into the stroke pathology and offer a mechanism of stem cell therapy, altogether providing the basis for developing an improved therapeutic strategy for stroke patients.

The overarching hypothesis of the present study is that mitochondrial dysfunction is an important factor in causing ganglion cell degeneration in retinal ischemia in vitro and in vivo models, and targeting this organelle via stem cell therapy may rescue the mitochondrial deficits and reverse pathology of retinal ischemia. The present study aims to fill the gap in our current
knowledge about the pathology underlying retinal ischemia and stroke, as well as in elucidating a potential mechanism of stem cell therapy. Understanding the role of mitochondrial dysfunction in retinal ischemia and whether mitochondria also play a key role the therapeutic mechanism underlying stem cell therapy will provide new insights into the pathology of retinal ischemia, as well as cerebral ischemia and other CNS disorders characterized by altered mitochondrial function and morphology, altogether forming the basis to develop an improved treatment for these diseases via mitochondria-mediated therapeutic strategies.
Chapter 2: Middle cerebral artery occlusion (MCAO) reduces blood flow to brain and eye and induces retinal ganglion cell loss

Note to Readers: Portions of this chapter have been previously published in “Nguyen H, Lee JY, Sanberg PR, Napoli E, Borlongan CV. An eye opener in stroke: Mitochondrial dysfunction and stem cell repair in retinal ischemia. In Press.”, and has been included with permission from the publisher.

2.1. Background

The most frequently used experimental models to study retinal ischemia involve intraocular pressure (IOP) or permanent ligation of posterior ciliary vessels/optic nerve. While these methods induce retinal ischemia, they do not reflect the dynamics of cerebral and retinal ischemic in stroke patients. Only few studies have examined the retinal ischemia in stroke models, such as MCAO. Here, we investigated whether retinal ganglion cell degeneration accompanied the MCAO stroke model.

2.2. Methods

2.2.1. Middle Cerebral Artery Occlusion

Adult male Sprague-Dawley rats (approximately 250g) were anesthetized by a mixture of 1–2% isoflurane in nitrous oxide/oxygen (69%/ 30%) via face mask. Body temperature was maintained at 37 ± 0.3 °C during the surgical procedures. The midline skin incision was made in the neck with subsequent exploration of the right common carotid artery (CCA), the external carotid artery, and internal carotid artery. A 4-0 monofilament nylon suture (27.0–28.0 mm) was
advanced from the CCA bifurcation until it blocked the origin of the middle cerebral artery (MCA). The contralateral CCA was temporally ligated for 10 minutes to ensure a consistent blood flow occlusion (including collaterals) to the brain. Animals were allowed to recover from anesthesia during MCAO. After 60 minutes of transient MCAO, animals were re-anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask and reperfused by withdrawal of the nylon thread. Animals receiving the sham operation were anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. A midline incision was made in the neck and the right CCA was isolated. The animals were then closed and allowed to recover from anesthesia.

2.2.2. Laser Doppler Blood Flow Measurement

Brain and eye retinal blood flow measurements were obtained using a laser Doppler (Perimed, Periflux System 5000). Each animal was placed under deep anesthesia during the measurement and the animal’s head was shaved for brain measurement. For measurement of brain perfusion, the laser Doppler probe was placed over the right frontoparietal cortical area supplied by the MCA. For measurement of eye perfusion, the laser Doppler probe was placed over the retina of the right eye. Measurements were made at baseline, during MCAO, and 5-minutes after reperfusion. Ophthalmic ointment was applied and the animals were closely monitored during their recovery from anesthesia.

2.2.3. Optic Nerve Measurement & Immunohistochemistry

On the day of tissue collection, the animals were euthanized with CO2 and perfused with 0.9% saline. The animals’ eyes were quickly harvested and the retina were isolated in ice cold phosphate-buffered saline (PBS), pH 7.4. The optic nerves and the retinas were fixed with 4%
paraformaldehyde (PFA; 158127; Sigma) in PB for 2 hours at 4°C. The tissues were washed with ice cold PBS for several times and kept in this solution at 4°C.

The retinas were embedded in 4% agarose and sectioned with a vibratome at 30 μm thickness in ice cold PBS. The tissues were incubated in blocking buffer (5% normal goat serum and 0.1% Tween 20 in PBS) for 3 hours at 4°C. The tissues were then incubated with NeuN (1:500; ab104225, Abcam) in blocking buffer at 4°C overnight. Thereafter, the tissues were washed three times with PBS followed by incubated with Alexa 488 secondary antibody (1:500) for 4 hours at 4°C. The tissues were further washed three times with PBS and mounted with anti-fade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories)

2.3. Results

2.3.1. MCAO Reduces Blood Flow to Brain and Eye

We initially investigated whether MCAO caused a reduction in blood flow to the brain, as well as to the eye. Laser Doppler was used to measure blood flow to brain and eye at baseline, during MCAO and 5-minute after reperfusion (Figure 2.1). At baseline, there were no significant differences between the control group and MCAO group in the laser Doppler measurements of ipsilateral hemisphere, contralateral hemisphere, or ipsilateral eye (311±23 and 316±87; 296±49 and 282±18; and 592±67 and 614±81, respectively, unpaired t-tests p(s)>0.05). During MCAO, there were significant differences between the control group and MCAO group in the percentage of blood flow to the contralateral hemisphere, ipsilateral hemisphere, and ipsilateral eye compared to the baseline (unpaired t-tests p(s)<0.05) (Table 2.1). The differences in the percentage of blood flow to the ipsilateral hemisphere and ipsilateral eye remained significant at
5-minutes after reperfusion between the control group and MCAO compared to the baseline readings (unpaired t-tests p(s)<0.05) (Table 2.1). Altogether, these results indicate that MCAO caused a significant reduction in blood flow to the eye which mirrored the blood flow reduction in the brain.

![Figure 2.1](image1.png)

Figure 2.1: MCAO reduced blood flow to brain and eye. Laser Doppler was used to measure blood flow to brain and eye at baseline, during MCAO, and 5-minute after reperfusion. (A) At baseline, there were no differences in blood flow measurements of the ipsilateral hemisphere, contralateral hemisphere, or ipsilateral eye between the control and MCAO group. (B) During MCAO, there were significant reductions in blood flow to brain and eye in the MCAO group compared to the control group. (C) The blood flow to ipsilateral hemisphere and ipsilateral eye remained reduced compared to the control at 5-minute after reperfusion. There was no significant difference in blood flow to the contralateral hemisphere between the MCAO and the control at 5-minute after reperfusion. Unpaired t-test(s) *** p<0.001; ** p<0.01; * p<0.05. Bar graphs represent mean ± SEM (n=6). Contra: contralateral; Ipsi: ipsilateral

Table 2.1: Percent blood flow during MCAO or 5-minute after reperfusion compared to baseline readings in different regions of control and stroke animals. Contra: contralateral; Ipsi: ipsilateral

<table>
<thead>
<tr>
<th></th>
<th>During MCAO vs. Baseline</th>
<th>5-minute After Reperfusion vs. Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MCAO</td>
</tr>
<tr>
<td>Contra. Hemisphere</td>
<td>89.4±27.0 %</td>
<td>29.2±17.0 %</td>
</tr>
<tr>
<td>Ipsi. Hemisphere</td>
<td>83.9±12.2 %</td>
<td>14.9±8.64 %</td>
</tr>
<tr>
<td>Ipsi. Eye</td>
<td>96.9±15.7 %</td>
<td>32.1±14.4 %</td>
</tr>
</tbody>
</table>

2.3.2. MCAO Induces Ganglion Cell Loss and Optic Nerve Degeneration at Day 3 and Day 14

We next examined whether the reduction in blood flow to the eye during MCAO caused significant ganglion cell loss and optic nerve degeneration in stroke animals. There were
significant reductions in ganglion cell at day 3 and day 14 in the ipsilateral eye compared to sham group (post-hoc test p(s)<0.001; Figure 2.2). In addition, there were significant reductions in the ipsilateral optic nerve width of stroke animals compared to the contralateral or sham animals at day 3 and day 14 (post-hoc test p(s)<0.001; Figure 2.3). ANOVA and post-hoc statistical analysis is summarized in Table 2.2.

Figure 2.2: MCAO induces ganglion cell loss in the retina at day 3 and day 14. Representative images of NeuN immunohistochemical staining for ganglion cells of the retina (A) horizontal (B) cross-section view. (C) Quantification graphs of ganglion cell count at day 3 and day 14. There were significant ganglion cell deaths in the ipsilateral eyes compared to contralateral eyes or
sham animals at day 3 and day 14. ANOVA with Bonferroni’s post-hoc test ***p<0.001. Bar graphs represent mean ± SD (n=6). Scale bar 50 µm. Contra: Contralateral; Ipsi: Ipsilateral

Figure 2.3: MCAO reduces optic nerve width at day 3 and day 14. Optic nerves were captured with bright field microscope near the optic nerve head. (A) Representative images of the optic nerves. (B) Quantification graphs of optic nerve width at day 3 and day 14. There were significant reductions in ipsilateral optic nerve width compared to contralateral or sham at day 3 and day 14. Contra: Contralateral; Ipsi: Ipsilateral. ANOVA with Bonferroni’s post-hoc test *p<0.05; ***p<0.001. Bar graphs represent mean ± SD (n=6). Scale bar 0.5 mm.

Table 2.2: ANOVA with Bonferroni’s post-hoc test of the optic nerve width and ganglion cell density between sham and MCAO animals at day 3 and day 14.

<table>
<thead>
<tr>
<th></th>
<th>Optic Nerve Width</th>
<th>Ganglion Cell Count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Contralateral</td>
</tr>
<tr>
<td>Day 3</td>
<td>( F(2,15)=13.97, )</td>
<td>( F(2,15)=13.97, )</td>
</tr>
<tr>
<td></td>
<td>( p=0.0004, )</td>
<td>( p=0.0004, )</td>
</tr>
<tr>
<td></td>
<td>post-hoc test ( p&lt;0.001 )</td>
<td>post-hoc test ( p&lt;0.001 )</td>
</tr>
<tr>
<td>Day 14</td>
<td>( F(2,15)=37.33, )</td>
<td>( F(2,15)=37.33, )</td>
</tr>
<tr>
<td></td>
<td>( p&lt;0.0001, )</td>
<td>( p&lt;0.0001, )</td>
</tr>
<tr>
<td></td>
<td>post-hoc test ( p&lt;0.001 )</td>
<td>post-hoc test ( p&lt;0.001 )</td>
</tr>
</tbody>
</table>
2.4. Conclusion

In this study, we examined whether MCAO stroke model induces retinal cell degeneration. We demonstrated that MCAO caused significant blood flow reductions to brain and ipsilateral eye. The blood flow was detected to be reduced in the ipsilateral hemisphere and the ipsilateral eye at 5-minute after reperfusion. This reduction in perfusion likely contributed to the significant ganglion cell death and optic nerve degeneration at day 3 and day 14. Our results support the idea that retinal ischemia can occur in conjunction with cerebral ischemia. Due to the anatomical juxtaposition of the ophthalmic artery to the MCA, blood flow to the ophthalmic artery is easily hindered in the event of MCAO stroke thereby causing retinal ischemia. Since MCAO is the most common form of stroke in human patients, the incidence of retinal ischemia occurring with cerebral ischemia is likely high. Retinal ischemia is a major predisposing factor of visual impairment and it is a shared pathology with other common ocular vascular diseases\textsuperscript{15-21}. Understanding the underlying mechanism of retinal ischemia may lead to better treatments for stroke patients with cerebral and retinal ischemia.
Chapter 3: Mitochondrial dysfunction is a key factor in retinal cell degeneration as evidenced by in vitro stroke model

Note to Readers: Portions of this chapter have been previously published in “Nguyen H, Lee JY, Sanberg PR, Napoli E, Borlongan CV. An eye opener in stroke: Mitochondrial dysfunction and stem cell repair in retinal ischemia. In Press.”, and has been included with permission from the publisher.

3.1. Background

As previously discussed, mitochondria play a significant role in cellular homeostasis, regulating apoptosis and modulation of inflammation. Therefore, it is important to assess the morphology and function of mitochondria in ischemic retinal cells. Here we utilized an in vitro stroke model to evaluate the role of mitochondrial dysfunction in retinal ischemia.

3.2. Methods

3.2.1. Retinal Pigmented Epithelium (RPE) Cell Culture

RPE (CRL-4000; ATCC) cells were cultured in Dulbecco’s Modified Eagle Media/F-12 (DMEM/F-12, 11320033; Gibco) containing 10% fetal bovine serum (FBS; FBS001; Neuromics) and 0.01 mg/ml hygromycin B (10687010; Gibco) in incubator (37°C humidified, with 5% CO₂, 95% air).
3.2.2. **Oxygen Glucose Deprivation (OGD): A Cell Culture Stroke Model**

A day prior to the experiment, RPE cells were seeded at $1.5 \times 10^5$ cells/well in 6-well plate. On the day of experiment, the media of the RPE cells was changed to Dulbecco’s phosphate-buffered saline (DPBS; 14040133; Gibco). The cells were placed in a sealed hypoxia incubator chamber (27310; StemCell Technologies) containing 95% N$_2$ and 5% CO$_2$) for 3 hours, mimicking the ischemic stroke. We chose 3 hours because based on our experience with RPE cells, 3 hours OGD provided 50-60% cell death. After the 3-hour period, fresh media was reintroduced and the cells were incubated in normoxia condition (37°C humidified atmosphere containing 5% CO$_2$) for 24 hours, which simulated the reperfusion phase in clinical setting.

3.2.3. **Mitochondrial Respiration Assay**

On the day of experiments, RPE cells were detached from cell culture plates and seeded to a Seahorse 96-well plate (101085-004; Agilent) coated with Poly-D-lysine (100µg/ml; P7886; Sigma) at 5.0 x 10$^4$ cells/well. The cells were immobilized by centrifugation method. Briefly, the Seahorse 96-well plate was centrifuged in swing bucket rotator with slow acceleration (4 on a scale of 9) to a max speed of 450 rpm with 0 brake. Afterwards, the plate orientation was reversed and centrifuged again to max speed of 650 rpm with 0 brake. To determine cellular oxygen consumption rate (OCR), the Seahorse extracellular flux analyzer XFe96 (102416; Agilent) was used in combination with sequential injection of various compounds (1 µmol/L oligomycin, 1 µmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 µmol/L Rotenone and Antimycin A). OCR measurements were performed according to the manufacturer’s protocol.
3.2.4. *Mitochondrial Network Analysis*

The RPE cells were stained with MitoTracker (500 nM; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Thereafter, the cells were carefully washed 3 times with DPBS, pH 7.4 to remove any excess dye residue. The cells were fixed with 4% PFA at room temp for 30 minutes. Finally, the cells were washed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories). Images were captured using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope and analyzed using ImageJ (NIH) with mitochondrial network analysis (MiNA) plugin.

3.2.5. *Mitochondria Live Cell Imaging*

The mitochondria of RPE cells were incubated with either mitochondrial membrane potential probe JC-1 (2 µg/mL; T3168; Invitrogen) or with MitoTracker (500 nM; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Next, the cells were carefully washed 3 times with PBS, pH 7.4 to remove any excess dye residue. Finally, the media was changed to DMEM without phenol red (21063029, Gibco). Live images were captured at using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope.

3.2.6. *Immunocytochemistry*

The RPE cells were rinsed 3 times with DPBS and then fixed with 4% PFA for 20 minutes at room temperature. The cells were rinsed again for 3 times and permeabilized with 0.3% Triton-X (X100; Sigma) for 5 minutes at room temperature. The cells were rinsed again a 3 times and incubated with blocking buffer containing 5% goat serum (50062Z; Invitrogen) at room temperature for 60 minutes. The cells were incubated with primary antibodies either rabbit
anti Ki67 (1µg/ml; NCL-Ki67P; LeicaBiosystems), at 4°C overnight. After rinsing several times with DPBS, the cells were incubated with Alexa Fluor 488 goat anti-mouse (Life Technologies) or Alexa Fluor 488 goat anti-rabbit for (Life Technologies) 60 minutes at room temperature. The cells were then rinsed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories).

3.3. Results

3.3.1. OGD Causes Mitochondrial Respiration Deficits in RPE Cells

In order to evaluate the mitochondrial respiration capacity after ischemia, RPE cells were exposed to either normal condition or OGD. RPE cells’ mitochondrial respiration was analyzed using Seahorse XFe96 extracellular flux analyzer (Figure 3.1). OGD caused significant reduction in the overall RPE cells’ mitochondrial respiration compared to control characterized by decreased basal respiration (13.8±2.3 and 25.1±1.7, respectively; unpaired t-test p<0.001), reduced spare respiratory capacity (13.1±3.0 and 24.8±2.0, respectively; unpaired t-test p<0.001), suppressed proton leak (1.4±0.7 and 5.0±0.7, respectively; unpaired t-test p<0.001), and lower ATP production (12.5±2.0 and 20.1±1.3, respectively; unpaired t-test p<0.001).
Figure 3.1: OGD causes significant mitochondrial respiration deficits. RPE cells’ mitochondrial respiration was analyzed using Seahorse XFe96 extracellular flux analyzer. The OCRs were measured at baseline and after sequential injections of compounds (1 µmol/L oligomycin, 1 µmol/L FCCP, 0.5 µmol/L Rotenone and Antimycin A). OGD causes significant reductions in basal respiration, spare respiratory capacity, proton leak and ATP production compared to control. Oligo: Oligomycin; FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Rot/AA: Rotenone and Antimycin A. Unpaired t-test ***p< 0.001. Bar graphs represent mean ± SEM control (n=11), OGD (n=12).

3.3.2. **OGD Alters RPE Cell’s Mitochondrial Network Morphology**

We further investigated the effect of OGD on mitochondrial network morphology. RPE cells’ mitochondrial network was analyzed using immunocytochemistry and ImageJ with MiNA plugin (Figure 3.2). The measured parameters were previously described. In brief, an individual is defined as mitochondria that do not have a junction and thus are either punctate or tubular depending on their circularity. Circularity (0 to 1) is a measurement of the shape of
mitochondria with 1 rated as being perfectly circular. A network is defined as mitochondria containing at least 1 junction exhibiting one or multiple branches. Mean network branches is the average number of branches per network while mean branch length is the average length of all rods/branches. Finally, footprint is the averaged total area with mitochondrial expression based on normalized intensity. Compared to control, OGD caused a significant reduction in total mitochondria footprint (38.9±12.3 and 52.4±21.0, respectively; unpaired t-test p<0.001), decreased in total individual mitochondria (58.8±20.1 and 83.1±26.8, respectively; unpaired t-test p<0.0001), decreased in number of network (6.59±2.80 and 9.00±3.97, respectively; unpaired t-test p=0.0087), and decreased in average branch length (0.79±0.10 and 0.91±0.13, respectively; unpaired t-test p<0.0001) while increased significantly the circularity of the mitochondria (0.53±0.07 and 0.45±0.06, respectively; unpaired t-test p<0.0001). Altogether these results demonstrate that OGD significantly altered the mitochondrial network morphology towards an impaired state, i.e., fragmented circular mitochondria.
Figure 3.2: OGD alters RPE cell’s mitochondrial network morphology. Representative images of RPE cells stained with MitoTracker (A) live cell imaging with bright field; scale bar 30 µm (B) immunocytochemical staining with DAPI; scale bar 10 µm. (C) Quantification of RPE cells’ mitochondrial network morphology. Compared to the control, OGD caused significant reductions in mitochondrial footprint, number of networks, total individual mitochondria, mean branch length and mean network branches while increased the circularity of mitochondria. Unpaired t-test *p<0.05; **p<0.01; ***p<0.001. Bar graphs represent mean ± SEM control (n=52), OGD (n=45).
3.4. Conclusion

In summary, we demonstrated that ischemia causes significant mitochondrial dysfunction characterized by an overall decreased in mitochondrial function and altered mitochondrial network morphology toward impaired state. Mitochondrial respiration assays revealed that OGD caused significant deficits to the mitochondrial bioenergetics machinery. In particular, after OGD, mitochondria were not able to produce as much ATP (ATP production) and had less ability to respond to energy stress (spare respiratory capacity). As previously discussed, mitochondria (and its ATP production) represent not only as the energy source of the cells, but also play a crucial role in regulating apoptosis. Therefore, maintaining sufficient level of ATP is crucial for cellular homeostasis especially in ischemic cells. In addition, OGD massively altered the mitochondria network morphology. As previously described, mitochondrial network is essential for maintaining mitochondrial functions. Mitochondrial network protects mitochondrial DNA integrity, improves respiratory capacity, exchanging materials, and response to energy demand or cellular stress. The overall morphology of mitochondrial network depends on the ratio of mitochondrial fusion and fission. In healthy cells, the balance ratio of mitochondrial fusion and fission allows mitochondria to display a tubular shape and form interconnected network. Conversely, low ratio of fusion to fission creates fragmented spherical mitochondria. In the present study, we demonstrated that OGD shifted the RPE cells’ mitochondrial network to impaired state i.e. fragmented circular mitochondria. For example, there were significant reductions in total number of mitochondria (footprint) and numbers of mitochondrial network in OGD cells compared to control. Within the mitochondrial network, there were less and shorter branches in OGD cells compared to control. Outside of the mitochondrial networks, there were less overall mitochondria (individuals) and they exhibited more spherical shape (higher
circularity). Taken together, ischemia altered the mitochondrial network to impaired state as evidenced by fragmented, spherical mitochondria.
Chapter 4: MSCs ameliorate retinal cell loss and rescue mitochondrial function possibly via transfer of mitochondria

Note to Readers: Portions of this chapter have been previously published in “Nguyen H, Lee JY, Sanberg PR, Napoli E, Borlongan CV. An eye opener in stroke: Mitochondrial dysfunction and stem cell repair in retinal ischemia. In Press.”, and has been included with permission from the publisher.

4.1. Background

As previously discussed, many studies have demonstrated the use of MSCs as promising cell-based therapy option for ischemic stroke. Here, we explored the potential therapeutic effects of MSCs on retinal ischemia utilizing in vivo and in vitro stroke models. We hypothesized that MSCs could attenuate the retinal cell loss caused by ischemia by restoring mitochondrial function and morphology. We also investigated a potential underlying mechanism in which MSCs transfer healthy mitochondria to injured retinal cells.

4.2. Methods

4.2.1. Middle Cerebral Artery Occlusion

Adult male Sprague-Dawley rats (approximately 250g) were anesthetized by a mixture of 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. Body temperature was maintained at 37 ± 0.3 °C during the surgical procedures. The midline skin incision was made in the neck with subsequent exploration of the right CCA, the external carotid artery, and internal carotid artery. A 4-0 monofilament nylon suture (27.0–28.0 mm) was advanced from the CCA
bifurcation until it blocked the origin of the MCA. The contralateral CCA was temporally ligated for 10 minutes to ensure a consistent blood flow occlusion (including collaterals) to the brain. Animals were allowed to recover from anesthesia during MCAO. After 60 minutes of transient MCAO, animals were re-anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask and reperfused by withdrawal of the nylon thread. Animals receiving the sham operation were anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. A midline incision was made in the neck and the right CCA was isolated. The animals were then closed and allowed to recover from anesthesia.

4.2.2. MSCs Cell Culture and Co-culture

MSCs (T4835;abm) were maintained with α-MEM (12561056; Gibco) supplemented with 20% FBS (FBS001; Neuromics), 1% penicillin/streptomycin (15140122; Gibco), 1% non-essential amino acids (11140050; Gibco), 1% GlutaMax-I (35050061; Gibco) in incubator (37°C humidified, with 5% CO₂, 95% air).

Prior to co-culture experiments, MSCs were seeded at 0.5 x 10⁵ cells/insert in cell culture inserts (353493, Falcon). After OGD, the RPE cells were co-cultured with MSCs by placing the inserts into the wells of the 6-well plate for 24 hours.

4.2.3. MSCs Transplantation

On the day of transplantation, MSCs were detached using TrypLE (Gibco 12604-021). Complete media was used to rinse the flask for maximizing cell yield. The MSCs were centrifuged at 300x g for 10 minutes. After the supernatant was aspirated, the cells were re-suspended in normal media. A small volume of cells were set aside for cell count. The cells were then centrifuged at 300x g for 5 minutes. Once the supernatant was aspirated, the cells were re-
suspended in final concentration of 4x10^6 cells/500 µL of sterile PBS. The animals were anesthetized and transplanted intravenously via the jugular vein with MSCs or with PBS only.

4.2.4. Immunohistochemistry

The retinas were embedded in 4% agarose and sectioned with a vibratome at 30 µm thickness in ice cold PBS. The tissues were incubated in blocking buffer (5% normal goat serum and 0.1% Tween 20 in PBS) for 3 hours at 4°C. The tissues were then incubated with NeuN (1:500; ab104225, Abcam) in blocking buffer at 4°C overnight. Thereafter the tissues were washed three times with PBS followed by incubated with Alexa 488 secondary antibody (1:500) for 4 hours at 4°C. The tissues were further washed three times with PBS and mounted with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories)

4.2.5. RPE Cell Culture

RPE (CRL-4000; ATCC) cells were cultured in DMEM/F-12 (11320033; Gibco) containing 10% fetal FBS (FBS001; Neuromics) and 0.01 mg/ml hygromycin B (10687010; Gibco) in incubator (37°C humidified, with 5% CO₂, 95% air).

4.2.6. OGD: A Cell Culture Stroke Model

A day prior to the experiment, RPE cells were seeded at 1.5x10^5 cells/well in 6-well plate. On the day of experiment, the media of the RPE cells was changed to DPBS; (14040133; Gibco). The cells were placed in a sealed hypoxia incubator chamber (27310; StemCell Technologies) containing 95% N₂ and 5% CO₂) for 3 hours, mimicking the ischemic stroke. We chose 3 hours because based on our experience with RPE cells, 3 hours OGD provided 50-60% cell death. After the 3 hours period, fresh media was reintroduced and the cells were incubated in
normoxia condition (37°C humidified atmosphere containing 5% CO₂) for 24 hours, which simulated the reperfusion in clinical setting.

4.2.7. Mitochondrial Respiration Assay

On the day of experiments, RPE cells were detached from cell culture plates and seeded to a Seahorse 96-well plate (101085-004; Agilent) coated with Poly-D-lysine (100µg/ml; P7886; Sigma) at 5.0 x 10⁴ cells/well. The cells were immobilized by centrifugation method. Briefly, the Seahorse 96-well plate was centrifuged in swing bucket rotator with slow acceleration (4 on a scale of 9) to a max speed of 450 rpm with 0 brake. Next, the plate orientation was reversed and centrifuged again to max speed of 650 rpm with 0 brake. To determine cellular OCR, the Seahorse extracellular flux analyzer XFe96 (102416; Agilent) was used in combination with sequential injection of various compounds (1 µmol/L oligomycin, 1 µmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 µmol/L Rotenone and Antimycin A). OCR measurements were performed according to the manufacturer’s protocol.

4.2.8. Mitochondrial Network Analysis

The RPE cells were stained with MitoTracker (500 ηmol/L; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Next, the cells were carefully washed 3 times with DPBS, pH 7.4 to remove any excess dye residue. The cells were fixed with 4% PFA at room temp for 30 minutes. Finally, the cells were washed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories). Images were captured using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope and analyzed using ImageJ (NIH) with mitochondrial network analysis (MiNA) plugin.
4.2.9. **Cell Viability Assay**

The RPE cells were incubated with calcein-AM (1 µmol/L; 4892010K; Trevigen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Bright green fluorescence was retained within living cells. The number of cells were counted using ImageJ (NIH) and averaged per field of view (FOV).

4.2.10. **Mitochondria Live Cell Imaging**

The mitochondria of RPE cells were incubated with either mitochondrial membrane potential probe JC-1 (2 µg/mL; T3168; Invitrogen) or with MitoTracker (500 ηmol/L; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Thereafter, the cells were carefully washed 3 times with PBS, pH 7.4 to remove any excess dye residue. Finally, the media was changed to DMEM without phenol red (21063029, Gibco). Live images were captured at using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope.

4.2.11. **Immunocytochemistry**

The RPE cells were rinsed 3 times with DPBS and then fixed with 4% PFA for 20 minutes at room temperature. The cells were rinsed again for a 3 times and permeabilized with 0.3% Triton-X (X100; Sigma) for 5 minutes at room temperature. The cells were further rinsed a 3 times and incubated with blocking buffer containing 5% goat serum (50062Z; Invitrogen) at room temperature for 60 minutes. The cells were incubated with primary antibodies either rabbit anti Ki67 (1µg/ml; NCL-Ki67P; LeicaBiosystems), DRP1 (70278; Life Technologies), or MFN2 (711803; eBioscience) at 4°C overnight. After rising several times with DPBS, the cells were incubated with Alexa Fluor 488 goat anti-mouse (Life Technologies) or Alexa Fluor 488 goat
anti-rabbit for (Life Technologies) 60 minutes at room temperature. The cells were then rinsed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories).

4.3. Results

4.3.1. MSCs Rescue Against Ganglion Cell Death in MCAO Stroke Model

We investigated the hypothesis that MSCs could rescue against the ganglion cell death caused by MCAO (Figure 4.1). Twenty-four hours after surgery, stroke animals received either MSCs or PBS via intravenous transplantation using the jugular vein. ANOVA revealed significant differences in ganglion cell count between groups (F(3,35)=28.71, p<0.0001).

Interestingly, transplantation of MSCs showed a trend toward a reduction in ganglion cell death at day 3 and a significant reduction in the ganglion cell loss at day 14 (post-hoc test p>0.05 and p=0.0026, respectively) compared to respective MCAO groups. There were no significant differences between MCAO group and MCAO+PBS group at day 3 and day 14 post stroke (post-hoc tests p(s)>0.05). Similar to previous experiment, we showed that MCAO caused ganglion cell loss at day 3 and day 14. Of interest, we demonstrated that intravenous transplantation of MSCs recued the ganglion cell death at day 14.
4.3.2. **MSCs Ameliorate OGD-induced RPE Cells Loss by Promoting Cell Proliferation**

We further investigated the observed therapeutic effect of MSCs under *in vitro* settings using OGD model (Figure 4.2). Cell viability and cell proliferation were assessed using calcein and Ki67 staining, respectively. ANOVA revealed significant differences in cell viability between groups (F(3,20)=45.75, p<0.0001), with OGD-RPE cells showing a significant decrease in cell viability compared to the control (119±70 and 1068±110, respectively, post-hoc test p<0.001). In contrast, co-culture with MSCs after OGD rescued the RPE cells’ viability compared to OGD group (512±327 and 119±70, respectively, post-hoc test p<0.01).
Additionally, ANOVA revealed significant differences in the Ki67/DAPI intensity ratio between groups (F(3,181)=47.03, p<0.0001) with OGD-RPE cells displaying a significant reduction in the intensity ratio compared to the control (2.27±0.73 and 2.83±0.47, respectively, post-hoc test p<0.01). Co-culture with MSCs after OGD increased the intensity ratio compared to OGD group (3.63±0.62 and 2.27±0.73, respectively, post-hoc test p<0.001). Interestingly, co-culture with MSCs with control did not increase the intensity ratio compared to control (2.63±0.55 and 2.83±0.47, respectively, post-hoc test p>0.05). Overall, the results demonstrate that MSCs prevented cell loss after OGD by promoting cell proliferation.

Figure 4.2: MSCs rescue against RPE cells loss caused by OGD by promoting cell proliferation. (A) Representative images of immunocytochemical staining of Ki67 (marker for cell proliferation). OGD produced a significant decrease in Ki-67/DAPI intensity. Co-culture with MSCs increased Ki-67/DAPI intensity after OGD. (B) Representative images of Calcein AM cell viability test. OGD induced a significant decrease in cell viability. Co-culture with MSCs rescued RPE cell death after OGD. (C) Quantification graphs of Ki-67/DAPI intensity and cell viability. ANOVA with Bonferroni’s post-hoc test **p<0.01; ***p<0.001. Bar graphs represent mean ± SEM. Scale bar 50 µm. OGD: Oxygen glucose deprivation; MSC: Mesenchymal stem cell.
4.3.3. **MSCs Attenuate RPE Cells' Mitochondrial Respiration Deficits Caused by OGD**

We further evaluated the effect of MSCs on the RPE cells’ mitochondrial respiration after OGD. RPE cells’ mitochondrial respiration was analyzed using Seahorse XFe96 extracellular flux analyzer (Figure 4.3). Co-culture with MSCs significantly rescued the overall mitochondrial respiration across all indices compared to OGD group as revealed by increased basal respiration (22.1±2.5 and 13.8±2.3, respectively; post-hoc test p<0.0001), increased spare respiratory capacity (27.4±3.3 and 13.1±3.0, respectively; post-hoc test p<0.0001), and increased ATP production (18.2±2.0 and 12.5±2.0, respectively; post-hoc test p<0.0001). Interestingly, we observed also a decreased in proton leak in the OGD group compared to the control or the OGD-MSCs (1.4±0.7, 3.9±0.7, and 5.0±0.7, respectively; post-hoc test p’s<0.0001). In summary, these results revealed that MSCs restored the mitochondrial respiration deficits caused by OGD. ANOVA analysis is summarized in Table 4.1.

![Mitochondrial Respiration](image)

**Figure 4.3:** MSCs ameliorate RPE cells’ mitochondrial respiration deficits caused by OGD. RPE cells' mitochondrial respiration was analyzed using Seahorse XFe96 extracellular flux analyzer.
The OCRs were measured at baseline and after sequential injections of compounds (1 µmol/L oligomycin, 1 µmol/L FCCP, 0.5 µmol/L Rotenone and Antimycin A). Co-culture with MSCs restored RPE cells’ mitochondrial basal respiration, spare respiratory capacity, proton leak, and ATP production compared to OGD. Oligo: Oligomycin; FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Rot/AA: Rotenone and Antimycin A. ANOVA with Bonferroni’s post-hoc test **p<0.01;***p<0.001. Bar graphs represent mean ± SEM control (n=11), OGD (n=12), OGD-MSC (n=12).

Table 4.1: ANOVA analysis of mitochondrial respiration parameters.

<table>
<thead>
<tr>
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<th>ANOVA Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Respiration</td>
<td>F(2,32)=82.03, p&lt;0.0001</td>
</tr>
<tr>
<td>Spare Respiratory Capacity</td>
<td>F(2,32)=85.51, p&lt;0.0001</td>
</tr>
<tr>
<td>ATP production</td>
<td>F(2,32)=55.69, p&lt;0.0001</td>
</tr>
<tr>
<td>Proton Leak</td>
<td>F(2,32)=80.77, p&lt;0.0001</td>
</tr>
</tbody>
</table>

4.3.4. **MSCs Restore RPE Cells’ Mitochondrial Networks that were Altered by OGD**

We also investigated whether MSCs could reverse the impaired RPE cells’ mitochondrial network morphology caused by OGD (Figure 4.4). The measured parameters were previously discussed in section 3.3.2. Compared to OGD group, co-culture with MSCs significantly increased the total mitochondrial footprint (45.8±14.4 and 38.9±12.3, post-hoc test p<0.0001), increased individuals of mitochondria (77.9±30.7 and 58.8±20.1, post-hoc test p=0.0046), increased the number of network (8.89±4.24 and 6.59±2.80, post-hoc test p=0.0180), increased mean average branches (6.02±2.10 and 4.74±1.27, post-hoc test p=0.002), and decreased circularity (0.48±0.07 and 0.53±0.07, post-hoc test p=0.0028), but not the average branch length (0.81±0.09 and 0.79±0.10, post-hoc test p>0.5). Altogether, these results demonstrate that MSCs rescued the mitochondrial network morphology which was altered by ischemia. ANOVA analysis is summarized in Table 4.2.

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Figure 4.4: MSCs restore RPE cells’ mitochondrial networks altered by OGD. (A) Representative images of RPE cells stained with MitoTracker. (B) Analysis and quantification of RPE cells’ mitochondrial network morphology. Co-culture with MSCs increased RPE cells’ number of mitochondrial networks, number of individual mitochondria, and number of branches but not average length of the branches compared to OGD. In addition, co-culture with MSCs decreased the circularity of RPE cells’ mitochondria compared to OGD. ANOVA with Bonferroni’s post-hoc test *p<0.05; **p<0.01; ***p<0.001. Bar graphs represent mean ± SEM Scale bar 10 µm.

Table 4.2: ANOVA analysis of mitochondrial network parameters.

<table>
<thead>
<tr>
<th></th>
<th>ANOVA Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Footprint</td>
<td>F(3,167)=8.312,p&lt;0.0001</td>
</tr>
<tr>
<td>Networks</td>
<td>F(3,167)=5.774,p=0.0009</td>
</tr>
<tr>
<td>Individuals</td>
<td>F(3,167)=9.955,p&lt;0.0001</td>
</tr>
<tr>
<td>Circularity</td>
<td>F(3,167)=13.36,p&lt;0.0001</td>
</tr>
<tr>
<td>Mean Branch Length</td>
<td>F(3,167)=10.64,p&lt;0.0001</td>
</tr>
<tr>
<td>Mean Network Branches</td>
<td>F(3,167)=4.580,p&lt;0.0001</td>
</tr>
</tbody>
</table>
4.3.5. MSCs Reduce RPE Cells' Mitochondrial Membrane Depolarization Induced by OGD and Mitochondrial Transfer as a Potential Mechanism of Action Mediating MSC Therapeutic Effects

RPE cells’ mitochondrial membrane potential was analyzed using JC-1 staining (Figure 4.5). ANOVA revealed significant differences between groups in the JC-1 red/green intensity ratio (F(3,119)=13.50, p<0.0001). Bonferroni’s post-hoc tests showed that OGD-RPE cells had significant decrease in the JC-1 red/green intensity ratio compared to the control RPE cells (0.94±0.59 and 1.63±0.49, respectively, p< 0.0001). Co-culture with MSCs significantly increased JC-1 red/green intensity ratio compared to OGD group (1.35±0.51 and 1.63±0.49, respectively, post-hoc test p<0.005). A decrease in JC-1 red/green intensity ratio indicated OGD-RPE cells’ mitochondria were unhealthy with depolarized mitochondrial membrane. Conversely, co-culture with MSCs increased the JC-1 red/green intensity ratio, thus significantly reducing the mitochondrial membrane depolarization.

It is worth noting that MSCs’ mitochondria were observed in both OGD-MSC and control-MSC groups. Confocal imaging revealed co-localization between MSCs’ mitochondria (blue) and JC-1 (red) indicating the transfer of functional mitochondria from MSCs to RPE cells (Figure 4.6). Indeed, analysis of co-localization of green/blue and red/blue signals is summarized in table 4.3. In particular, there is a positive correlation between JC-1 red signal and MSCs’ mitochondria blue signal (Pearson’s coefficient = 0.65 ± 0.08) with M1 & M2 coefficients of 0.57 ± 0.16 and 0.54 ± 0.18, respectively. This mitochondrial transfer phenomenon was confirmed with immunocytochemical staining as evidenced by deposition of MSCs’ mitochondria within the boundary of RPE cells (Figure 4.7). Furthermore, correlational analysis revealed that there was a positive correlation between the percentage of cells with transferred
mitochondria and the cell viability ($r=0.9402$, $n=35$, $p<0.0001$ with a $R^2=0.8840$; Figure 4.8).

In summary, these results indicate that MSCs reduced RPE cells’ mitochondrial membrane depolarization caused by OGD in part possibly via transfer of MSCs’ functional mitochondria.

Figure 4.5: MSCs reduce RPE cells’ mitochondrial membrane depolarization caused by OGD. RPE cells’ mitochondrial membrane potential was analyzed using JC-1 staining. Representative images of JC-1 dye (red and green) and transferred MSC’s mitochondria (blue). Bar graph represents red/green (healthy/unhealthy) intensity ratio of JC-1 staining. OGD-RPE cells displayed a significant decrease in the JC-1 red/green intensity ratio compared to the control RPE.
cells. Co-culture with MSCs significantly increased JC-1 red/green intensity ratio compared to OGD. ANOVA with Bonferroni’s post-hoc test **p<0.01; ***p<0.001. Bar graphs represent mean ± SD. Scale bar 10 µm.

Figure 4.6: MSCs transferred mitochondria to ischemic RPE cells. Confocal imaging revealed co-localization between MSCs mitochondria (blue) and JC-1 (red, arrows) indicating the transfer of functional mitochondria from MSCs to RPE cells. Scale bar 10 µm.
Table 4.3: Co-localization analysis of JC-1 intensity and MSCs’ mitochondria.

<table>
<thead>
<tr>
<th></th>
<th>Control-MSC (n=36)</th>
<th>OGD-MSC (n=57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green &amp; Blue Pearson's Coeff.</td>
<td>0.90 ± 0.02</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>M1 Index</td>
<td>0.49 ± 0.13</td>
<td>0.53 ± 0.21</td>
</tr>
<tr>
<td>M2 Index</td>
<td>0.70 ± 0.16</td>
<td>0.61 ± 0.17</td>
</tr>
<tr>
<td>Red &amp; Blue Pearson's Coeff.</td>
<td>0.60 ± 0.07</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>M1 Index</td>
<td>0.59 ± 0.12</td>
<td>0.57 ± 0.16</td>
</tr>
<tr>
<td>M2 Index</td>
<td>0.60 ± 0.17</td>
<td>0.54 ± 0.18</td>
</tr>
</tbody>
</table>

Figure 4.7: MSCs’ mitochondria were detected in RPE cells after OGD. MSCs’ mitochondria were separately stained with Mitotracker prior to co-culture with RPE cells. Confocal images of RPE cells with DAPI (blue), β-tubulin (red), and MSCs’ mitochondria stained with Mitotracker (green). MSCs’ mitochondria were detected within the boundaries of RPE cells. Scale bar 10 μm.
Figure 4.8: Correlational analysis between mitochondria transfer and cell viability. There was a positive correlation between the percentage of cells with transferred mitochondria and number of viable cells (n=35).

4.3.6. **MSCs Repair RPE Cells’ Mitochondrial Dynamics via Mfn2 after OGD but not Drp1**

We further investigated the deleterious effect of OGD and therapeutic effect of MSCs on mitochondrial dynamic proteins Mfn2 and Drp1. Immunocytochemical assay of Mfn2 revealed that there were significant differences between groups ([F(3,307)=15.65, p<0.0001; Figure 4.9]). OGD significantly reduced the expression of Mfn2 compared to the control (post-hoc test p<0.0001). Co-culture with MSCs significantly restored the expression of Mfn2 compared to the OGD-RPE group (post-hoc test p<0.0001). Immunocytochemical assay of Drp1 revealed that there were significant differences between groups ([F(3,80)=9.747, p<0.0001; Figure 4.10]). OGD significantly increased the expression of Drp1 compared to the control (post-hoc test p<0.05), but co-culture with MSCs did not significantly restore the expression of Drp1 to normal level.
These results show that OGD altered the mitochondrial dynamic proteins Mfn2 and Drp1, while co-culture with MSCs normalized the expression of Mfn2, but not Drp1.

Figure 4.9: MSCs normalize RPE cells’ mitochondrial dynamics via Mfn2 after OGD. Representative images of Mfn2 expression (left columns), DAPI (middle columns) and merged (right columns). OGD caused a significant decrease in Mfn2 expression. Co-culture with MSCs significantly increased the Mfn2 expression compared to OGD. ANOVA with Bonferroni’s post-hoc test *p<0.05; **p<0.01; ***p<0.001. Bar graphs represent mean ± SD. Scale bar 50 µm.
Figure 4.10: MSCs did not restore RPE cells’ Drp1 expression level after OGD. Representative images of Drp1 expression (left columns), DAPI (middle columns) and merged (right columns). OGD caused a significant increase in Drp1 expression. Co-culture with MSC did not decrease the Mfn2 expression compared to OGD. ANOVA with Bonferroni’s post-hoc test * p<0.05; **p<0.01; ***p< 0.001. Bar graphs represent mean ± SD. Scale bar 50 µm.
4.4. Conclusion

In the present study, we demonstrated that intravenous transplantation of MSCs significantly rescued retinal ganglion cell death in MCAO stroke model at day 14. MSCs promoted cellular proliferation by increasing Ki-67 expression and rescued RPE cell loss in OGD \textit{in vitro} stroke model. Of note, that co-culture MSCs with control RPE cells did not increase Ki-67 expression, suggesting that injured or “help-me” signals might be necessary for MSCs to exert therapeutic effects. Furthermore, MSCs attenuated the mitochondrial respiration deficits caused by ischemia and rescued the mitochondrial network morphology. In particular, co-culture with MSCs increased the overall mitochondrial footprint, increased the number of mitochondrial networks, and decreased the mitochondrial circularity. These observations indicate that MSCs returned the mitochondria to healthier state with tubular mitochondria and more intricate network. In addition, JC-1 staining showed that MSCs rescued the mitochondrial membrane depolarization caused by OGD. Restoring the mitochondrial membrane potential is essential for the function of mitochondria. Interestingly, we demonstrated that the mitochondrial transfer phenomenon occurs between MSCs and RPE cells. Immuncytochemistry and JC-1 live cell imaging revealed that MSCs transferred healthy mitochondria to injured RPE cells. Indeed correlational analysis showed that the higher number of RPE cells with transferred mitochondria correlated with higher cell viability. Additionally, OGD disrupted mitochondrial dynamics proteins Mfn2 and Drp1 which play important role in regulating mitochondrial fusion and fission processes, respectively. Co-culture with MSCs after OGD normalized the expression of Mfn2 but did not restore the Drp1 expression level to normal. A preferential restoration of the mitochondrial fusion over fission dynamics was evident in our data, in that MSCs promoted mitochondrial fusion which might facilitate the integration of the transferred MSCs’
mitochondria into RPE cell’s mitochondrial network. Furthermore, this observation seems to support the mitochondrial network analysis of RPE cells after co-culture with MSCs. Specifically, compared to OGD group, while there was no significant difference in the average length of the network branches, there was significant increase on average more branches per network in co-culture with MSCs group. A possible explanation is that MSCs transferred and facilitated the integration of mitochondria into the network by promoting mitochondrial fusion. Taken together, the results demonstrated that MSCs promoted cell survival rescued mitochondrial dysfunction and shifted the mitochondria to a normalized and functional state (i.e. tubular mitochondria and intricate network) possibly via transfer of healthy mitochondria.
Chapter 5: Discussion

Stroke remains one of the leading causes of death and disability in the United States \(^1\). Recent advances in stroke awareness and treatment protocols have reduced the overall mortality rate \(^1\). Yet, stroke patients continue to experience a wide range of functional disability with severe emotional and financial consequences \(^1\). Furthermore, the current limited stroke treatment options focus in restoring blood flow with narrow therapeutic window targeting the early stage of stroke pathology \(^2-5\). To date, physical therapy is the only option to mitigate the disabilities associated with chronic stroke. Moreover, visual impairment is a common disability in stroke patients, but largely an ignored stroke consequential symptom. Visual impairment affects the ability for stroke patients to exhibit functional recovery and improved quality of life \(^10, 11\). Retinal ischemia is a major underlying cause of visual impairment in stroke and it is a shared pathology with other common ocular vascular diseases such as glaucoma, diabetic retinopathy, and CRAO \(^20-27\). There are many risk factors associated with both cerebral ischemia and CRAO \(^27, 28\). Due to the anatomical proximity of the ophthalmic artery and the MCA, ischemic stroke resulting from MCAO is likely to block blood flow to the ophthalmic artery \(^47\). Therefore, treatment regimens that address cerebral ischemia and retinal ischemia represent as appealing strategies to ameliorate visual impairments and improve outcome for stroke patients. The underlying mechanism of retinal ischemia is not fully understood despite pathological similarities with cerebral ischemia. In particular, the role of mitochondrial dysfunction in retinal ischemia remains to be elucidated. Mitochondria not only serve as the powerhouse of the cell but also play important role in maintaining cellular homeostasis, regulating apoptosis and modulating inflammation \(^117-123\).
Thus, mitochondrial dysfunction which is a key mediator in cerebral ischemia pathology is likely a principal pathological trigger for retinal ischemia. Therapies designed in combating mitochondrial dysfunction as a primary target might be beneficial for retinal ischemia, stroke and other CNS disorders with retinal ischemia pathology. Since its discovery a few decades ago, stem cell therapy has been investigated for neurological disorders such as stroke \(^{48-51}\) and ocular degenerative diseases such as glaucoma, age-related macular degeneration, and diabetic retinopathy\(^{47, 52-55}\). However, despite years of scientific advancement and limited clinical trials, the complicated underlying mechanism of cell-based therapy remains elusive and not fully understood. This lack of in-depth understanding of mechanistic underpinnings of stem cell therapy corresponds to the fundamental gap in our knowledge before stem cell therapy can be widely utilized in large-scale clinical settings. The overarching focus of this work is to advance the understanding of mitochondrial dysfunction as a major mechanism of cell death in stroke-induced retinal ischemia and explore the therapeutic mechanism of stem cell to restore mitochondrial function thereby providing the basis for cell-based therapies for retinal ischemia, stroke and other impaired mitochondria-related CNS disorders.

First, we investigated whether MCAO stroke model induces retinal ischemia and retinal degeneration. We monitored blood flow to brain and eye at baseline, during MCAO and 5-minute after reperfusion utilizing laser Doppler. We observed that the perfusion profile of the eye differs from the brain at baseline, characterized by a much higher blood flow. It might due to the higher number of vasculatures in the retina than the brain that are detectable by laser Doppler \(^{244, 245}\). Furthermore, the difference in collateral blood vessels between retina and brain may also contribute to the distinct perfusion profiles. However, under the stroke pathological condition, we demonstrated that MCAO caused similar patterns of significant reduction in blood flow to
brain and eye. This reduction in blood flow persisted at 5-minute after reperfusion in the ipsilateral hemisphere and ipsilateral eye compared to control. Since the perfusion of the eye mirror similarly to the brain, this opens potential application for stroke responders to quickly assess the severity of certain type of stroke while transporting patients to facilities with imaging capabilities. The reduction in blood flow in the eye corresponds to retinal ischemia which was likely the cause of significant retinal ganglion cell loss and optic nerve degeneration at day 3 and day 14. Our data extend the ischemic retinal injury time course to 14 days compared to previous studies which showed the retinal ischemia mirrors the cerebral ischemia up to 3 days post stroke \(^{241, 242, 246}\). Similar to cerebral ischemia, time is of the essence and the earlier that retinal ischemia is detected and treated the better the outcome \(^{247-250}\). However, despite the lack of collaterals, retinal cells are generally considered to be more resistant to ischemic insults than the brain \(^{25, 27}\). Clinical studies suggest that the effective time window for central retinal artery occlusion with intravenous tPA administration could be up to 6 – 6.5 hours \(^{251, 252}\). In animal study, due to the use of different models and species, there are some inconsistencies regarding therapeutic window which is summarized in a review \(^{25}\). As noted above, the anatomical juxtaposition of the ophthalmic artery to the MCA, which similarly gets occluded resulting in retinal ischemia in the event of MCAO may contribute to the different reperfusion profiles. Because of the unique “dosing” of vasculature and collaterals (or lack thereof) in the brain and the retina, the discordant reperfusion profiles will likely affect the stem cell distribution or mitochondrial transfer in these tissues. Notwithstanding the subtle differences in blood flow and collaterals between the brain and the eye, it is still important to diagnose and treat retinal ischemia as early as possible similar to cerebral ischemia.
Next, we examined the hypothesis that mitochondrial dysfunction plays a key role in retinal ischemia pathology using *in vitro* stroke model. Here, we measured the mitochondrial respiration using Seahorse extracellular flux analyzer XFe96 and utilized immunocytochemistry to analyze mitochondrial network morphology between control and OGD RPE cells. Mitochondrial respiration capacity provides direct insights into the ability of mitochondria to perform the critical function of generating energy for the cells. Similarly, mitochondrial network morphology provides understanding to the state of mitochondria. For example, healthy mitochondria have elongated tubular shape and are capable of forming elaborate networks with multiple branches. In contrast, impaired mitochondria display fragmented and spherical shape morphology. Indeed, our data demonstrated that OGD caused significant deficits to mitochondrial respiration characterized by routine electron transport chain measurements such as decreased in ATP production and decreased in spare respiratory capacity (the ability to respond to energy demand). Furthermore, ischemic injured RPE cells contained fewer total mitochondria, decreased number of network, and increased circularity. Interestingly, mitochondria in impaired cells localized adjacent to the nucleus which might be a cellular defensive mechanism to protect the DNA. Altogether, these data indicate that mitochondrial dysfunction is closely associated with stroke pathology.

We investigated the hypothesis that transplantation of MSCs is a potential therapeutic option for retinal ischemia by ameliorating the mitochondrial dysfunction. In animal study, we demonstrated that intravenous transplantation of MSCs at day 1 post stroke showed a trend toward rescue at day 3 and significantly decreased retinal ganglion cell loss at day 14 compared to control and vehicle-treated animals. This histopathological benefit of MSCs supports the restorative effects of cell therapy in retinal ischemia. We showed that MSCs ameliorate RPE cell
loss caused by OGD by promoting proliferation in an *in vitro* stroke model. The possibility also exists that MSCs also maintained mitochondrial function in spared cells (e.g., ischemic cells in the penumbra), which would suggest that MSCs mount both protective and reparative effects \(^{209-216}\). We further examined the effect of MSCs on mitochondrial function and network morphology. Co-culture with MSCs rescued against mitochondrial respiration deficits caused by ischemic injury. Furthermore, MSCs improved the mitochondrial network morphology of OGD RPE cells. In addition, we assessed the mitochondrial membrane potential using JC-1 dye in order to further evaluate the overall mitochondrial state. Healthy mitochondria will have a JC-1 red/green ratio higher than 1, while unhealthy mitochondria will have a ratio less than 1. Indeed, the results showed that MSCs attenuated the mitochondrial membrane depolarization caused by ischemia. Most importantly, we demonstrated that MSCs transferred healthy mitochondria to injured RPE cells which likely contributed to the observed mitochondrial functional recovery. A logical question arises if the transferred mitochondria would be able to integrate with the endogenous mitochondrial network. To this end, we examined the mitochondrial dynamics via the expression of Mfn2 and Drp1 proteins. Mfn2 and Drp1 are important proteins responsible for fusion and fission of mitochondria, respectively. Under normal condition, mitochondrial fusion augments mitochondrial function by sharing substances and mtDNA across the tubular network; while mitochondrial fission facilitate clearance of damaged proteins and DNA \(^{158,160,161}\). On the other hand, under pathological condition, the imbalance between mitochondrial fusion and fission leads to apoptosis \(^{162,163,166}\). The discrepancy between expression of fusion and fission proteins may be critical for the onset of fragmented spherical mitochondria morphology observed after ischemia. Our data concur with previous studies demonstrating that after ischemic insults, there is an upregulation of Drp1 and a
downregulation of Mfn2. Interestingly, MSCs significantly restored Mfn2 but not Drp1 expression level. As we briefly discussed in section 4.4, this set of data supports the notion that MSCs promoted the integration of mitochondria into network via upregulating mitochondrial fusion. Such normalized fusion dynamics may mediate the restored mitochondrial networks in cells co-cultured with MSCs which displayed on average more branches compared to non-treated group.

The novel data presented in this work add to the body of scientific knowledge implicating the role of mitochondria in stroke pathology and its treatment. However, this dissertation, like most scientific inquiry has limitations and presents as many questions as answers. The focus of our study is on the mitochondria’s role in retinal ischemia in ganglion cells since they are neuronal cells that make connections with the brain. However, we did not investigate other cell types such as photoreceptors, amacrine cells, and residential immune cells of retina, which are equally vulnerable to ischemic injury. In addition, in the present study, we did not explore visual behavior tests to evaluate whether the observed cell loss correlate with visual deficits. Including visual behavior tests in a future study would further strengthen the significance and clinical relevance of these findings. While this study focused in mitochondrial dysfunction and proposed the transfer of mitochondria as a mechanism of MSCs, other mitochondria-relevant organelles (e.g., endoplasmic reticulum, microvesicles, and exosomes) which may be altered in stroke pathology and treatment are potential targets that need to be considered in future investigations. Future studies could focus on the contribution of these mitochondrial components to the pathologic and treatment outcomes. As previously discussed, the other established mechanisms mediating stem cell therapy such as by stander effects including secretion of growth factors, anti-inflammatory, anti-oxidative, and anti-apoptotic substances, are as equally important as the
transfer of mitochondria. Future studies could investigate the potential synergistic effects if any between these stem cell mechanisms of action. The transfer of mitochondria from MSCs to ischemic retinal cells to amend mitochondrial impairment begs the question of signaling pathways involved in the release and uptake of mitochondria. In the context of in vivo stroke, injured cells release “help me signals” which could be used to guide the migration of stem cells to the target area such as brain or retina. Recent literature has offered equally compelling evidence of other potent mechanisms associated with mitochondrial transfer (e.g. TNTs, EVs, gap junctions, and cell fusion). In the present study, we employed the non-contact co-culture system between MSCs and retinal cells. Hence, in our case, the mitochondrial transfer occurs likely through extracellular vesicles. This raised certain questions. How do these vesicles form? What is the minimal and optimal size of these vesicles containing mitochondria? How far can the released vesicle travel? Are there other molecules in these vesicles that could contribute to the observed benefits? Addressing these questions will further elucidate the underlying mechanism. On this note, utilizing rho-like MSCs that do not contain mitochondria, isolated mitochondria, or changing the pore size of the co-culture membrane are some of the alternative approaches to answer these questions. Moreover, studies that focus in the profile and characteristics of the released vesicles are likely to be beneficials for the understanding of these vesicles. These approaches also delineate the contribution of mitochondrial transfer compared to other well-know mechanisms of cell therapy. A better understanding of the underlying mechanism of mitochondrial transfer will also lay the foundation for developing molecules and compounds which facilitate the transfer as well as strengthen the function of transferred mitochondria. Optimizing the timing of MSCs transplantation, routes of delivery as well as dosage would help to maximize the functional benefits of mitochondrial-based therapy.
Moreover, despite the shortcomings of tPA, it remains standard treatment of care for stroke. Future studies should investigate the potential therapeutic effects of tPA with and without stem cells which could yield insightful knowledge in developing effective mitochondrial treatment. Notwithstanding these limitations, we demonstrated that MCAO and OGD induced retinal ischemia, associated with mitochondrial dysfunction. Treatment with MSCs rescued against retinal cell loss, likely through stem cell transfer of healthy mitochondria and subsequent restoration of mitochondrial function, network morphology, and dynamics.

In summary, we used a combination of in vitro cell culture and in vivo rat models to examine the role of mitochondria dysfunction in stroke-related retinal ischemia and whether stem cells could repair the mitochondria and rescue the ischemic retinal cells. Our results demonstrated that both MCAO and OGD stroke models produced consistent retinal ischemia accompanied by massive alterations in retinal cells’ mitochondrial respiration, network morphology, and dynamics and treatment, which were reversed by stem cell treatment. From the translational standpoint, because stem cell therapy has reached clinical trials for stroke, a better understanding of its mechanism of action may optimize the safe and effective application of this treatment for stroke patients. That cell-free, organelle-based approach such as mitochondria transplantation may suffice as a clinical product for stroke may circumvent many of the logistical limitations (e.g., immune response) associated with cell therapy. From the basic science viewpoint, the appreciation of mitochondria as the powerhouse of the cell highlights the role of this specific organelle in maintaining cell homeostasis and function under normal and pathological conditions.

This work is built upon the foundation of many experts in the field of stroke and stem cell therapy in the quest to find a better treatment options for stroke and relevant vascular diseases.
Through the efforts of leading experts in the field such as my mentor, I am proud and confident that the work presented here moves us even just by a little closer to a better treatment option for diseases that affect millions of people worldwide.
References


Appendix


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