2013

Effect Of Oxidative Stress On Human Brain Vascular Pericytes

Rasha Mohamed
North Carolina Agricultural and Technical State University

Effect of Oxidative Stress on Human Brain Vascular Pericytes

Rasha Mohamed

North Carolina A&T State University

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Chemical, Biological and Bio engineering

Major: Bioengineering

Major Professor: Dr. Donghui Zhu

Greensboro, North Carolina

2013

School of Graduate Studies
North Carolina Agricultural and Technical State University
This is to certify that the Master's Thesis of

Rasha Mohamed

has met the thesis requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2013

Approved by:

Dr. Donghui Zhu Major Professor	Dr. Jenora T. Waterman Committee Member
Dr. Matthew B. McCullough Committee Member	
Dr. Stephen B. Knisley Department Chair	Dr. Sanjiv Sarin Dean, The Graduate School

Biographical Sketch

Rasha Mohamed was born on October 12th in Alexandria, Egypt. She graduated from Alexandria University of Medicine in 1996. She has continued her education and is a candidate for the Masters of Science degree in Bioengineering at North Carolina Agricultural and Technical State University.

Dedication

I would like to dedicate this work to my mother Fatma al-Sharkawy, who was encouraging me to complete my degree but passed away before I had finished.

Acknowledgements

I would like to acknowledge my advisor Dr. Donghui Zhu. Thank you for your constant encouragement and confidence in my capabilities. I would also like to thank Dr. Jenora Waterman, for her generous assistance and for allowing me to work in her lab. I learned so much from my professors and I am grateful that they took the time to work with me.

Table of Contents

List of Figures

Abstract

The occurrence of neurodegenerative diseases (NDs) increases with extended life expectancy. The rise in elderly populations is resulting in an increased incidence of cognitive impairment, and a high risk for developing dementia. Oxidative stress is implicated in the pathogenesis of a number of neurovascular disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD). It has also been linked to disruption of blood brain barrier (BBB) in hypoxic-ischemic injury. As part of the neurovascular unit, pericytes are inevitably subjected to insult from oxygen and nitrogen radicals, generated in the central nervous system (CNS), leading to cell dysfunction or damage.

In this research, we studied the effect of oxidative stress on cerebro-vascular pericytes cellular function and determined how oxidative stress changes pericytes protein expression, including F-actin, α-actin, platelet-derived growth factor receptor beta (PDGFR-β), tropomyosin and myosin. In vitro, human brain vascular pericytes cell cultures were exposed to hydrogen peroxide (H_2O_2) to mimic the cerebral ischemic condition then evaluate the cytotoxicity by MTT viability assay, LDH assay and quantity of cellular morphology deterioration. Intracellular Proteins expression level was examined by quantitative immunefluorescent microscopy. Proteins were labeled with specific primary antibodies and corresponding secondary antibodies, conjugated with different fluorescent probes. Results from cytotoxicity tests suggest that when we exposed the pericytes cells to 100 and 200 μM for 1 hour, it showed the toxic effect of H_2O_2 on the pericytes cells without killing the cells. Our data reveals that after mild oxidative stress, pericytes protein expression intensity increased, changed position and moved toward the cell membrane while sometimes forming cellular protrusion connecting cells together. These results indicate that oxidative stress can affect pericytes cell contraction and cell movement.

CHAPTER 1

Introduction

1.1 Oxidative Stress

Oxidative stress is a state where excess reactive oxygen species (ROS) overwhelm the endogenous antioxidant system. Oxidative stress is involved in pathogenesis of many neurovascular disorders such as Alzheimer's disease (AD), Parkinson's disease and multiple sclerosis (Coyle & Puttfarcken, 1993). Oxidative stress also causes disruption of blood brain barrier (BBB). Moreover, oxidative stress causes injury to neurons and alters astrocyte functions (Robb & Connor, 1998). ROS induces pericytes contraction (Attwell et al., 2010).

1.2 Blood Brain Barrier (BBB)

Blood brain barrier is a complex structure that regulates the flow of the ions, macromolecules and leukocytes into and out of the brain. BBB is formed by brain micro-vascular endothelial cells (BMVEC), pericytes and astrocytes. BBB disruption, induced by infection, trauma or autoimmunity disease, initiates the parenchymal inflammation and facilitates the leukocytes entry into the brain parenchyma via par-cellular route (Schenkel et al., 2004). Immune cells cross BBB through the trans-cellular route (Doulet et al., 2006). Migration of leukocytes is mediated by leukocytes –endothelial interaction and chemokine secretion by BMVEC, astrosytes and microgalia (Cullere et al., 2005; Yang et al., 2005). Loss of BBB integrity occurs in many neurological diseases, such as Alzheimer's disease, Parkinsonism disease, ischemia and multiple sclerosis.

1.3 Human Brain Microvascular Pericytes

In 1873, French scientist Charles- Mari Benjamin Rouget described pericytes cells and named them Rouget cells (Rouget, 1873). Fifty years later, Zimmermann renamed these cells

pericytes due to their anatomical location in the endothelium (Dalkara et al., 2011). Cerebrovascular pericytes are small cells located in the outer layer of the brain capillary, between the endothelial cell layer and the parenchyma. They are separated from the parenchyma by the basal lamina (Franket al., 1987). Recruitment of pericytes to the vessel wall in the embryonic brain regulates the development of the cerebral microcirculation and the blood brain barrier (BBB). This is mediated by endothelial secreted platelet derived growth factor-B (PDGF-B) and platelet derived growth factor receptor beta (PDGFR-β) interaction (Hellstorm et al., 1999; Tallquistet al., 2003). Pericytes play an important function in angiogenesis, vessel stabilization, endothelial cell regulation and maintenance of the blood-brain barrier (Bell et al., 2010; Herman et al., 1985). Pericytes also show macrophage-like activity (Lai & Kuo, 2005). They are also contractile cells, involved in regulation of capillary blood flow in response to vasoactive agents and neural activity (Dore-Duffy et al., 2006; Hamilton et al., 2010).

1.4 Neurodegenerative Diseases and Oxidative Stress

Neurodegenerative diseases are diseases of the brain and spinal cord nerve cells. They may be presented as function loss, which lead to ataxia or sensory dysfunction and dementia (Mattson, 2004). Oxidative stress (OS) leading to free radicals attack on neural cells. This free radicals play a significant role in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple Sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (McCord, 2000). Toxicity of free radicals contributes to protein and DNA injury, inflammation, tissue damage and subsequent cellular apoptosis (Fridovich, 1999). The incidence of neurodegenerative diseases (NDs) increases with extended life expectancy. Alzheimer's disease (AD) is the most common neurodegenerative disease and it affects approximately 4.5 million Americans and 16 million people worldwide (Gandhi et al., 2012).

1.5 Thesis Outline

Chapter 2 was a literature review of oxidation stress and its role in neurodegenerative diseases. The chapter also included a literature review of pericytes, anatomical and histological structure and function, role in normal activity and role in disease states. Chapter 3 discused methods and materials used to create and evaluate results obtained from this study. Chapter 4 displayed all results observed in the study regarding effect of oxidative stress in pericytes. Lastly, chapter 5 presented a discussion of the results revealed and suggested future studies.

CHAPTER 2

Literature Review

2.1 Oxidative Stress

Oxygen is essential for any cell life. However, the metabolism byproduct of oxygen produces reactive oxygen species (ROS), which are toxic to cells. Oxidative stress is the state of imbalance that results from a production of (ROS) that exceeds the ability of antioxidant defense mechanisms (Poon et al., 2004). Early in life, 1-2% of O_2 consumed is converted to ROS, but this percentage increases in an aged brain due to the reduction of antioxidants and the low regenerative capacity of an aged brain (Lepoivre et al., 1994).

2.1.1 Reactive oxygen species (ROS). ROS refers to the reactive molecules containing oxygen. These include free radicals and are able to produce oxidative changes in the cells (Migliore et al., 2009; Zhang et al., 2008). ROS sources include 1) mitochondrial electron transport chain (Barja, 2004); 2) Proximal fatty acid metabolism; 3) Cytochrom P-450 reactions (Beckman et al., 1998; Harman, 1992). ROS include superoxide (O_2•−), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•). ROS lead to neurodegeneration by modulating the function of biomolecules, which results in oxidative damage to macromolecules, as lipids, proteins and nucleic acids (Poon et al., 2004; Stadtman, 2004). ROS attack glial cells and neurons leading to neuronal damage (Gilgun-Sherki et al., 2001).

2.1.2 Oxidative stress and neurodegenerative disease. Oxidative stress plays an important role in the etiology of many late onset degenerative diseases. Aging is the most important risk factor for the common neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Lin et al.(2006) studied the relation between age and neurodegenerative

diseases. They found that mitochondria are important regulators of cell death. Oxidative stress leads to mitochondrial DNA mutation and dysfunction.

2.1.2.1 Alzheimer's disease (AD). Alzheimer's disease (AD) is the most common neurodegenerative disease and it affects approximately 4.5 million Americans and 16 million people worldwide. By 2050, it is projected that approximately 13 million people in the USA and 100 million people worldwide will be affected by this disease (Hebert et al., 2003).

Alzheimer's disease is a progressive brain disease that slowly destroys memory and thinking skills. AD patient may develop behavioral changes with time. Many studies show evidence of the toxic effect of oxidative stress products on AD brain. Mecocci et al. (1994) reported that oxidative stress affects DNA in the parietal cortex of the AD patient, mainly mitochondrial DNA and, to a lesser extent, nuclear DNA. Hensley et al. (1995) observed that protein oxidation occurs in elderly people with or without AD, but it is more severe in AD patients. Studies by Palmer et al. (1994) and Marcus et al. (1998) show increased lipid peroxidation in the brains of AD patients, mainly in the temporal lobe. Perry et al. (2003) and Nelson et al. (2009) found that, approximately, 90% of AD patients have olfactory dysfunction. Evidence of oxidative stress has been observed in the olfactory epithelium of AD patients. There is lipid peroxidation in the nuclear and cell membrane of olfactory neurons and epithelial cells. Neurofibrillary tangles formation and amyloid β peptide deposition lead to extensive cell loss and olfactory dysfunction. There are many studies about Alzheimer's disease pathogenesis and its relation to oxidative stress. Lovell et al. (1995) reported that Alzheimer's disease is characterized by progressive neuronal loss and aggregation of protein extracellular and intracellular, forming extracellular amyloid β (βA) plaques, and forming intracellular tau tangles. ROS lead to increased levels of malondialdehyde and 4-hydroxynonenal in the brain and cerebrospinal fluid of AD patients.

2.1.2.2 Parkinson's disease (PD). Parkinson's disease is a common adult-onset neurodegenerative disorder. It is the second most common neurodegenerative disease, characterized by a degeneration of the dopamine cell in the substantia nigra and aggregation of the protein α-synuclein. Oxidative stress plays a major role in the initiation and progression of PD. Many studies report that oxidative stress leads to lipid, protein, and DNA damage. A study by Dalfó et al. (2005) showed that polyunsaturated free fatty acids concentration in the substantia nigra is decreased but levels of lipid peroxidation markers, such as malondialdehyde and 4-hydroxynonenal, are increased. These can react with proteins to impair cell viability. Brown et al. (2004) reported that reactive nitrogen species lead to nitration and nitrosylation of certain proteins. Moreover, research by Bender et al. (2006) showed that oxidative stress leads to increased deletions in mitochondrial DNA in the dopaminergic neurons in PD substantia nigra. Many studies have focused on the relation between PD-related genes (PINK1, DJ-1, Parkin) and mitochondrial function. Lev et al. (2008)**,** for example, reported that DJ-1 in mitochondria has an antioxidant effect and loss of its function leads to oxidative stress. Researchers (Crompton et al., 2002; Giorgio et al., 2010) have also studied the PINK1gene, which is a mitochondrial kinase. Studies reported that PINK1 deficiency leads to impairment of mitochondrial calcium efflux and mitochondrial calcium overload. Reaction between calcium and ROS leads to an opening of the mitochondrial permeability transition pore (PTP). When opened, PTP allows molecules to escape to cytosol, causing cell death. The reaction between PINK1act and Parkin was investigated in several studies (Geisler et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Such studies reported that PINK1act with Parkin to regulate the clearance of damaged mitochondria via autophagy. Mutations in either Parkin or PINK1 lead to a failure of mitophagy and an

accumulation of dysfunctional mitochondria, which causes more production of ROS and more oxidative stress to the neuron.

2.1.3 Mechanisms of oxidation stress. Oxidation stress occurs when ROS exceeds the ability of antioxidant defense mechanisms. ROS can be increased due to mitochondrial dysfunction. Direct activation of NADPH oxidase can also cause oxidation stress.

2.1.3.1 ROS production by mitochondrial dysfunction. The mitochondria have been identified as a major source of ROS. It produces free radicals due to a defect in the electron transport chain. This electron transport chain is a complex enzymatic system consisting of 5 distinct phases: complex 1 (NADH dehydrogenase), complex 2 (succinate dehydrogenase), complex 3 (ubiquinol–cytochrome-c reductase), complex 4 (cytochrome-coxidase), and complex 5 (ATP synthase). Rodrigo, Miranda, and Vergara (2011) reported that mitochondria is a major source of cellular (O_2•−) production from Complex I and from Complex III by the transfer of one electron to O_2. According to this theory, inhibition of mitochondrial complex will induce oxidative stress. Also, mitochondria produce ROS via the enzyme family of monoamine oxidase (MAO), which bind to the outer mitochondrial membrane and catalyze the oxidation of biogenic amine neurotransmitters, such as, nor epinephrine, dopamine, and serotonin. MAO-B catalyzes the oxidation of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) to (MPP^+), which inhibits mitochondrial complex I (Lotharius et al., 2000; Smith et al., 1997).

2.1.3.2 ROS production via NADPH oxidase. A lot of researchers studied the NADPH oxidase and its role on oxidative stress in Alzheimer's disease. Bianca et al. (1999) reported that Amyloid β leads to the direct activation of NADPH oxidase in rat microglial cells and human phagocytes. Furthermore, Abramovet al. (2003) reported that Amyloid β activate

NADPH oxidase through increased calcium influx into astrocytes. The activation of NADPH oxidase leads to the generation of oxidative stress.

2.2 Brain Microvascular Pericytes

2.2.1 What are pericytes? They are contractile cells found on almost all capillaries, small arterioles and venules walls. Rouget C (1873) was the first scientist to describe pericytes and termed them Rouget cells. Zimmermann K (1923) coined the term Pericytes because of their location around microvessels. Frank et al. (1987) described the pericytes and their location, clarifying that pericytes are tiny cells situated on the outer layer of the vessel, between the endothelial cell layer and the parenchyma. They are surrounded by basal lamina, which separates them from endothelial cells and parenchyma. Shepro et al. (1993) reported that pericytes send out primary projections along the vessel in each direction, then from this projection, they send secondary and, maybe, tertiary processes projections around the vessel. Additionally, Morphological studies of pericytes showed that pericytes have a heterochromatic nucleus (D. E. Sims, 1986). The cytoplasm of pericytes contains mitochondria, ribosomes, Golgi apparatus, lysosome, rough and smooth endoplasmic reticulum (David E. Sims, 2000). Pericytes have primary processes along the vessel wall and secondary processes, which go around the vessel wall (Krueger et al., 2010).

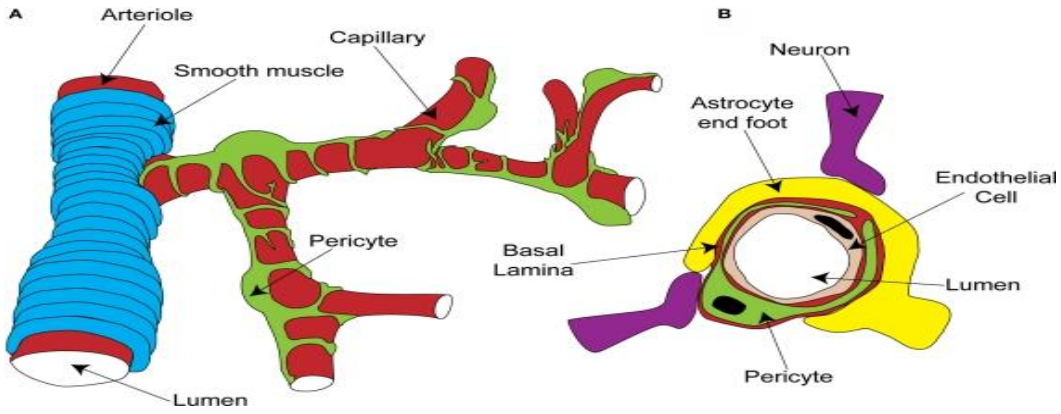


Figure 1. Capillary neurovascular unit (Hamilton et al., 2010).

2.2.2 Pericytes functions. Many studies have investigated the function of pericytes and their role in normal physiology. Pericytes play an important role in the maintenance of the blood-brain barrier, vessel stabilization, endothelial cell regulation and angiogenesis (Hirschi et al., 1996; Krueger et al., 2010; C. H. Lai et al., 2005). Pericytes show macrophage-like activity (W. E. Thomas, 1999). They also act as precursors for several different cell types, forming neurons and glia in the CNS (Bonkowski et al., 2011; Dore-Duffy et al., 2006). Early researchers thought that pericytes are contractile cells because of their prevascular location and that they may play a role in the regulation of microcirculation blood flow (Dore, 1923). Researchers such as Frank et al. (1987) and Shepro et al.(1993) studied pericytes in the central nervous system and they found that there are more pericytes per endothelial cell in CNS more than anywhere else. This indicates that pericytes might play a particularly specific role in the nervous system.

2.2.2.1 Regulation of capillary blood flow. Capillary pericytes are contractile cells, which can respond to many vasoactive stimuli. Electron microscopic studies reported that pericytes have microfilament around the nucleus and within the primary and secondary processes. These microfilaments contain actin, myosin, or tropomyosin (Hamilton et al., 2010; David E. Sims, 2000). Such results correspond with immunoelecton microscopy and immunohistochemical studies of retinal and brain pericytes (Bandopadhyay et al., 2001). Dehouck et al. (1997) found that activation of endothelin- 1 receptors lead to an elevation of the intracellular calcium concentration. This in turn, leads to vasoconstriction of cultured capillary pericytes. Recently, more studies on rat and mouse were performed to confirm that pericytes are contractile cells that can regulate blood flow. Peppiatt et al. (2006) stimulated retinal and cerebellar rat pericytes with ATP and noradrenalin and detected the contraction of capillary

pericytes with differential interference contrast microscopy. In a study by Fernández-Klett et al. (2010), the authors reported that vasoactive stimuli in an intact adult mouse brain lead to the contraction of cortical capillary pericytes. Constrictions were more prominent at pericytes bodies, which reflected changes in pericytes contractile tone. This lead to changes in the capillary diameter associated with significant changes in blood flow. Furthermore, Webb (2003) reported that smooth muscle contraction or relaxation depended on intracellular calcium concentration. Elevation of intracellular calcium concentration leads to activation of myosin light chain kinase. This generates contraction by phosphorylating the myosin light chain (MLC) and prompting its interaction with α-SMA. Low calcium levels leads to the relaxation of the smooth muscle.

2.2.2.2 Pericytes and regulation of BBB. The blood-brain barrier (BBB) is considered a physical barrier. BBB is formed by the endothelial cell, extracellular matrix, astrocytes and pericytes. The role of pericytes in the regulation of BBB is emphasized in the study byArmulik et al. (2010). An in vivo study of adult mouse pericytes reported that the close interaction between the endothelial cells and pericytes are important for BBB development. The study also reported that the capillaries permeability depends on pericytes number. Pericytes deficiency increases the permeability of the BBB to water and molecules. Daneman et al. (2010) reported that pericytes induce the formation of tight junctions and inhibit the expression of molecules that increase vascular permeability. Al Ahmad et al. (2009) reported that in many cerebrovascular diseases, pericytes can be detached from the vessel wall, leading to impairment in BBB and resulting in decreased tight junction expression and increased pinocytic activity. Quaegebeur et at. (2010) reported that pericytes in healthy conditions release molecules to brain endothelial cells in order to maintain the BBB integrity. Pericytes also secure normal perfusion and vasoregulation. BBB

lose integrity when pericytes lost under pathological condition lead to decreased vessel vasoreactivity, hypoperfusion and hypoxia. Leakage of neurotoxic substances leads to abnormal endothelial junctions, transcytosis, and astrocyte polarization.

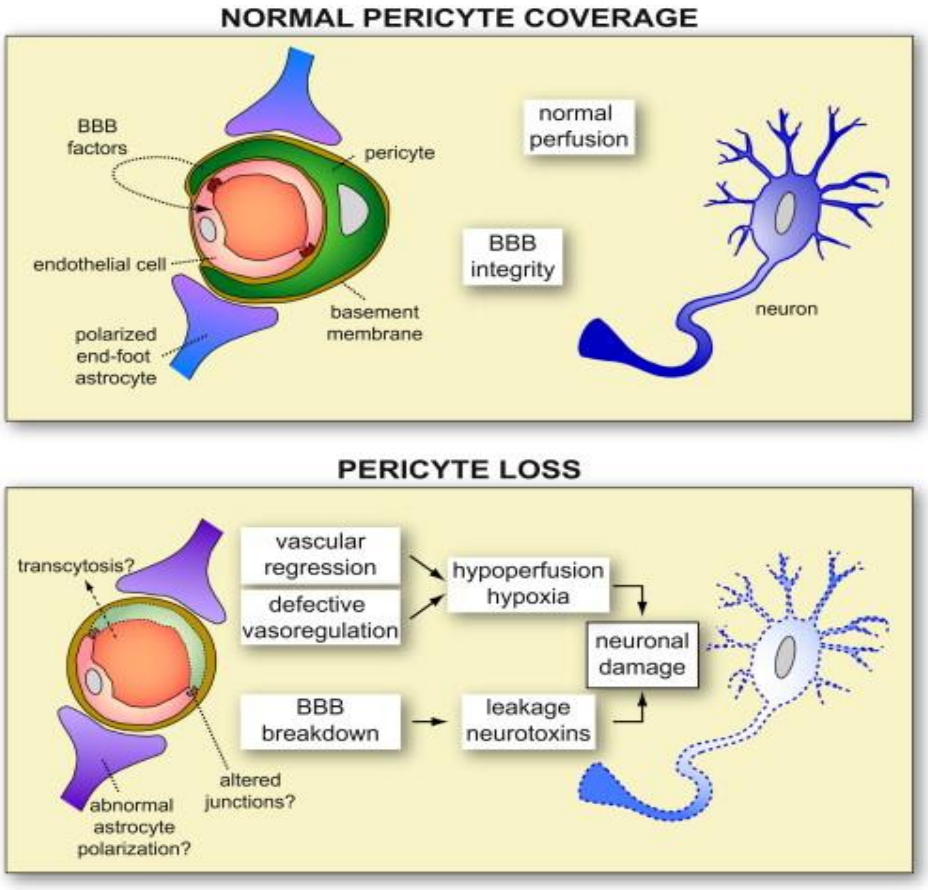


Figure 2. Roles of pericytes in BBB integrity and brain perfusion (Quaegebeur et al., 2010).

2.2.2.3 Pericytes and angiogenesis. Angiogenesis is the process of new blood vessels formation. This process requires two types of cells, endothelial cell and pericytes (Gerhardt & Betsholtz, 2003). A study by Lindahl et al. (1997) investigated the reaction between the endothelial cells and pericytes cells. He reported that endothelial cells secrete PDGF-B, which binds to PDGFR-β receptor in pericytes. This interaction is essential for the presence of pericytes in the vessels wall. Abramsson et al. (2007) studied the role of Heparin sulfate (HS) in pericytes

recruitment, using two mouse models with altered HS biosynthesis. The authors concluded that pericytes recruitment requires sufficient amounts of HS to maintain PDGF-B and activate PDGF receptor β (PDGFR-β) signaling.

2.2.3 Role of pericytes in pathological conditions. Pericytes damage has been linked to several pathological conditions. Various researchers (M. Hellstrom et al., 2001; Kamouchi et al., 2004; Puro, 2007) found that the contractile activity of pericytes is regulated by intracellular Ca^{2+} concentration. Many factors may cause an uncontrolled rise in intracellular Ca^{2+}. ROS may be one of these factors. A study by Yemisci et al. (2009) states that ROS produced by endothelium and astrocyts on the microvessel wall cause pericyte contraction. Mathiisen et al. (2010) reported that mitochondrial pericytes may be a good source for ROS under pathological conditions. An in vitro study by Kamouchi et al. (2007) showed that ROS causes sustained increase in Ca^{2+} in cultured human brain pericytes. Moreover, a study by Yemisci et al. (2009) performed in an intact mouse brain showed that ROS causes pericyets contraction. So, excessive ROS and Ca^{2+} dysregulation lead to the dysfunction of pericytes and the loss of signaling between pericytes and surronding cells.

2.2.3.1 Alzheimer's disease. Pericytes play an important role in pathogenesis of Alzheimer's disease. Wilhelmus et al. (2007) reported that β-amyloid deposition around capillaries in Alzheimer's patient brain is toxic to pericytes. He reported also that pericytes may be directly involved in β-amyloid clearance across the blood-brain barrier. Many researchers investigated the causes of β-amyloid clearance insufficiency. Weller et al. (2008) reported that this may be due to overproduction of β-amyloid or due to decreased functioning of the clearance pathways. (Niwa et al., 2001; T. Thomas et al., 1996) reported that the β-amyloid react with the endothelial cells and release a superoxide radicals, which lead to vasoconstriction, endothelial

cell damage and a decrease in cerebral blood flow in larger blood vessels. This also causes a decrease in the response of cerebral blood vessels to neuronal activity or to endothelium-dependent vasodilators.

2.2.3.2 Diabetic retinopathy. Visual loss due to retinopathy is one of the most common complications of diabetes mellitus. Retinopathy is characterized by acellular capillaries, microaneurysms, hemorrhages, macular edema and angiogenesis (Cogan et al., 1961). Retinal capillaries have a higher number of pericytes more than in any other vascular bed in the body (Frank et al., 1987; Shepro et al., 1993). Loss of pericytes in the retinal microvessels is the most important feature of diabetic retinopathy. Interaction between pericytes and endothelial cells is important to keep pericytes on retinal capillaries. Platelet-derived growth factor-B (PDGF-B) is produced by endothelial cells, while its receptor, PDGFR-β is expressed on pericytes. Lindhal et al. (1997) reported that PDGFR-β knockout mice showed microvascular pericytes loss and developed numerous capillary microaneurysms that ruptured later. Pericytes are important to maintain retinal capillary stability and prevent microaneurysm formation. Geraldes et al. (2009) addressed that there is a possible link between Diabetes and PDGFR- β deficiency. He reported that the high glucose levels lead to activation of protein kinase C-δ and decrease signaling pathway in pericytes, which leads to PDGFR-β phosphorelation, causing pericytes apoptosis and loss. He also reported that activation of protein kinase (p38αK) leads to generation of ROS that cause activation of nuclear factor κB (NF-κB) and activation of SHP-1(Src homology-2 domain-containing phosphatase-1). This inhibits PDGFR-β and stops the reaction between the endothelial cell and pericytes. Ejaz et al. (2008) spotted that protein kinase C-δ lead to activation of NF-kB and pericytes apoptosis.

2.2.3.3 Cerebral ischaemia. Cerebral ischaemia refers to the interruption of cerebral blood flow at the capillary level. Peppiatt et al. (2006) studied the role of pericytes in retinal and cerebral ischaemia. The authors reported that capillary pericytes contracted during retinal ischaemia and transient cerebral ischaemia. They found that pericytes can control capillary diameter in the whole retina and cerebellar slices. Electrical stimulation of retinal pericytes started a capillary constriction. Superfused ATP in retina or nor adrenaline in cerebellum resulted in constriction of capillaries by pericytes. This suggests that pericytes are probably modulators of blood flow in response to changes in neural activity. Yemisci et al. (2009) reported that contracted pericytes induced segmental narrowing of the capillary lumen, leading to clogged circulation. Further, del Zoppo et al. (2003) reported that capillary contraction induced by pericytes contraction may delay the passage of blood cells and promote their aggregation with fibrin.

Several electron microscopic studies reported that after ischaemia, pericytes migrate away from the vessel wall (Gonul et al., 2002; Melgar et al., 2005; Takahashi et al., 1997). This migration started at the time of hypoperfusion and continued for at least 12–24 hours. Pericytes that did not migrate showed cytoplasmic changes and degeneration. This suggests that migration may protect the pericytes from death or cell death prevent cell from migration.

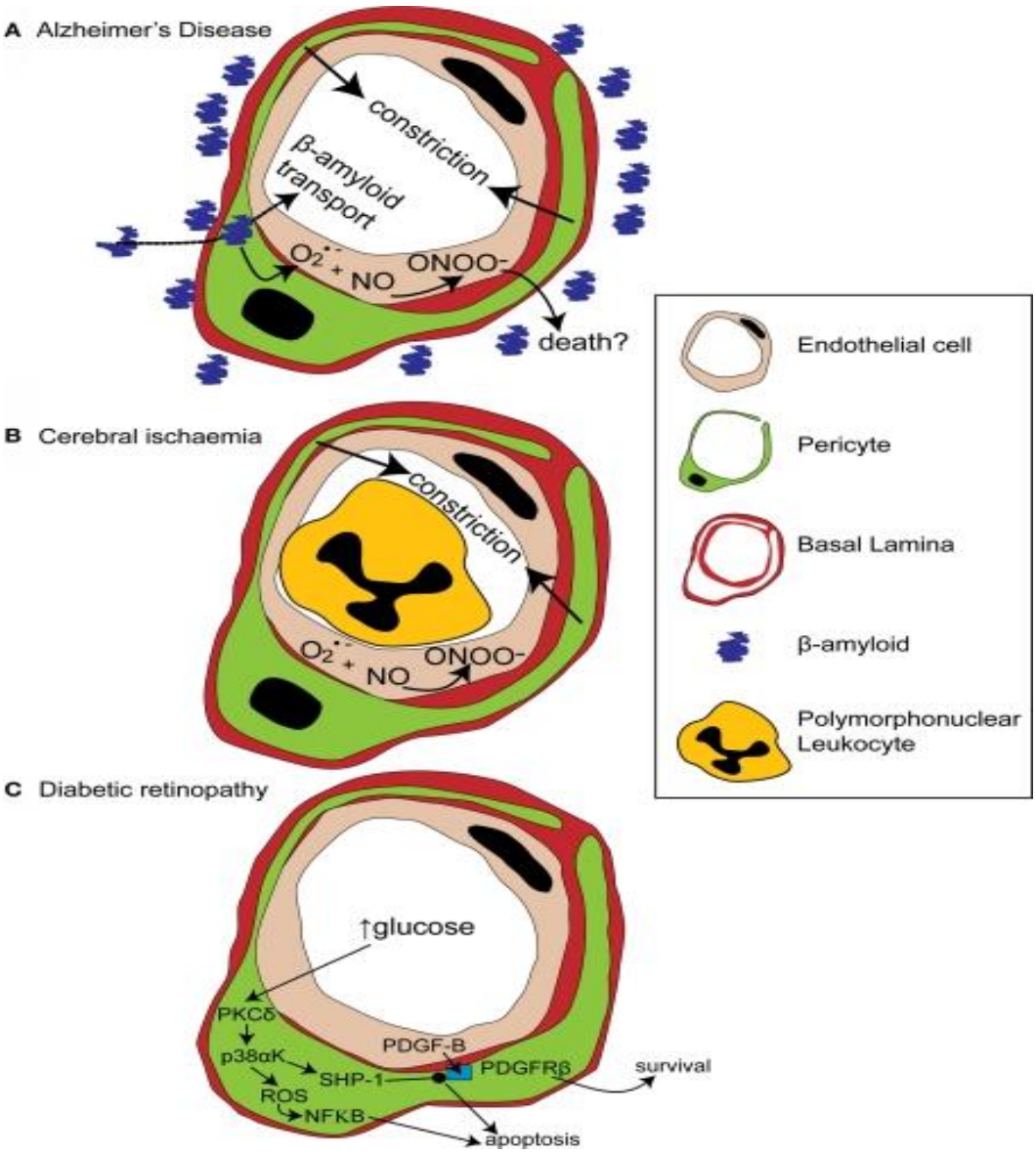


Figure 3. Pericytes in disease states (Hamilton el al., 2010).

A) In Alzheimer's disease: β-amyloid, which aggregates around blood vessels, is toxic to pericytes and produces superoxide ($O_2•^-$) leading to vessel contraction.

B) In cerebral ischaemia: capillary blockade by polymorphonuclear leukocytes, generation of reactive oxygen species and capillary constriction lead to failure of capillaries reperfusion.

C) In diabetic retinopathy: high glucose levels prompt pericytes apoptosis via activation of protein kinase C δ (PKCδ) and activation of protein kinase (p38αK). This generates ROS that

cause activation of nuclear factor κB (NF-κB) & activation of SHP-1(Src homology-2 domain-containing phosphatase-1), which inhibits PDGFR-β and stops the reaction between the endothelial cell and pericytes.

2.3 Purpose

The purpose of this thesis is to study the effects of oxidative stress on human brain vascular pericytes cellular function and determine how oxidative stress changes the pericytes protein expression. The literature review has identified that ROS are able to produce oxidative changes in the cells (Migliore et al., 2009; Zhang et al., 2008)**.** Oxidative stress may alter intracellular calcium load in pericytes by interfering with mitochondria and calcium pump, which may account for the sustained calcium rise and pericytes contraction (Kamouchi et al., 2007; Yemisci et al., 2009). Pericytes contraction cause capillary contraction and may delay the passage of blood cells and promote their aggregation with fibrin (del Zoppo et al., 2003). Therefore, there is an urgent need to illustrate what and how oxidative stress may alter pericytes cellular function in details. The hypothesis of this study is that oxidative stress affects cerebro-vascular pericytes cellular function and leads to increased pericytes protein expression level, which promotes pericytes contractility causing capillary contraction. The objectives are to: 1) Evaluate the effect of oxidative stress on cerebro-vascular pericytes cellular function; recognize the toxic concentration, and determine how long it took to show the toxic effect of H_2O_2 on the human brain vascular pericytes cells without killing these cells through in vitro tests, including MTT viability assay, LDH assay and Cell Morphology. 2) Determine how oxidative stress changes pericytes protein expression, including F-actin, α-actin, PDGFR-β, tropomyosin and myosin.

CHAPTER 3

Methodology

Human brain vascular pericytes (HBVP) cells were tested in this study by exposing them to H_2O_2 to investigate the toxic effect of H_2O_2 on the cell. Cultured cells are often exposed to H_2O_2 concentration ranging from 100 µM to 1000 µM. Tests included cytotoxicity and intracellular proteins expression level. The cytotoxicity assay measured the toxic effect of H_2O_2 on human brain vascular pericytes cells (HBVP) with an MTT viability assay, LDH assay and quantity of cellular morphology deterioration. The intracellular proteins expression level is examined by quantitative immunofluorescent microscopy.

3.1 Measuring H_2O_2 Concentration

To prepare fresh 225 mM stock H_2O_2, first dissolve 230 µl of 30% H_2O_2 in 9.77 ml of sterile water then prepare 1:30 dilution and read the absorbance at 215 nm three times and average them. To calculate H_2O_2 concentration, use the average absorbance and Beer –Lambert Law ($A=\varepsilon bc$), $c = (A/\varepsilon b)$*dilution factor & $C_1V_1= C_2V_2$ and prepare different concentrations to determine which concentration is toxic to the cells without killing them (Dr. Jenora Waterman, personal communication).

3.2 Cytotoxicity Assay

Cytotoxicity tests are one method used to assess the toxic effect of H_2O_2 on cellular function. Cell membrane integrity is the most common way to measure cytotoxic effects on cells. There are many different ways to measure cytotoxicity, including 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (Promega, Madison, WI) and Lactate Dehydrogenase (LDH) assay (Roche, Indianapolis, IN).

3.2.1 Media preparation. Pericytes Medium (PM) is a medium created for optimal growth of normal human vascular pericytes in vitro. It is a sterile, liquid medium, containing essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals and a low concentration of fetal bovine serum (2%). This medium is bicarbonate-buffered and has a pH of 7.4 when incubated with an atmosphere of 5% CO_2 /95% air. The medium is prepared to provide a balanced nutritional environment that selectively promotes proliferation and growth of normal human vascular pericytes in vitro **(**ScienCell Research Laboratories). PM consists of 500 ml of basal medium, 10 ml of fetal bovine serum (FBS), 5 ml of pericytes growth supplement (PGS), and 5 ml of penicillin/streptomycin solution (P/S). Thaw PGS, FBS and P/S solution at 37ºC then add PGS, FBS and P/S solution into a basal medium in a sterile field stored at 4ºC.

3.2.2 Cell culture. HBVP cells purchased from ScienCell Research Laboratories (Carlsbad, CA) were cultured in a T-75 poly-L-lysine coated flask (overnight coating) with Pericytes Medium (PM), 2% of fetal bovine serum (FBS), 1% penicillin / streptomycin solution (P/S) and 1% of pericytes growth supplement (PGS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were first seeded from liquid nitrogen into poly-L-lysine coated T-75 flasks. Fresh (PM) media was added after 24 hours to the flasks then cells were cultured for a minimum of 72 hours before a media change or subculture. Once the monolayer reached subconfluence (80%), cells were subcultured by trypsinization. Cells were used for experiments at passage 3 or 4. To prepare the cells for cytotoxicity tests, cells were seeded in poly-L-lysine coated 96-well plates at 2×10^4 cells/200 µl per well and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 72 hours to allow attachment and reach a high confluence (70%). The medium was then replaced with a different concentration of H_2O_2 at different periods of exposure to determine the

concentration and how long it takes to show the toxic effect on cells without killing the cell. Cell culture plates were observed under an optical microscope. Experiments were run in triplicate to ensure repeatability and decrease variation. The optical microscope was used to capture images at different magnifications, including 10X, 20X and 40X to observe and compare cell morphologies. Images were captured from each well and compared to the control well.

3.2.3 Optical microscope images of cell morphologies. In order to inspect the morphologies of human brain vascular pericytes cells after exposure to different concentrations of H_2O_2 at different period of time, an EVOS optical microscope (AMG, Grand Island, NY) was used. The EVOS microscope, which is a transmitted light, digital-inverted microscope, was applied with phase contrast settings to conduct a comparison of the morphologies of treated and normal untreated pericytes cells.

3.2.4 Cell viability assay (MTT). Cell viability testing is a type of cytotoxicity testing used to measure the toxic effect of substance or material on cells via viability. Viability is the relative growth rate of treated cells compared to control cells, which are not treated. Viability is significant, because it quantitatively measures the effect of substance exposure on cell growth. There are various tests to quantitatively measure cytotoxicity. These tests include MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromid), LDH (lactate dehydrogenase) and BrdU (5-bromo-2'-deoxyuridine). Each test uses different biomarkers to determine the mechanism of cell death. Those biomarkers are ATP, LDH and proteases.

In this study, MTT colorimetric assay was completed to calculate the number of viable cells in proliferation or cytotoxicity assays using The CellTiter 96 AQueous One Solution. The MTT assay reagent contains a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-

coupling reagent (phenazine ethosulfate; PES). The MTT assay is dependent on the reduction of yellow tetrazolium salt MTT to purple formazan crystals in the mitochondria of living cells. This cellular reduction is most likely achieved by NADH and NADPH (Promega, 2012).The test should include a blank containing complete culture medium without cells to account for any background noise from the solution when running the assay. The formazan crystals formed are solubilized in tissue culture media and the resulting purple solution is quantified using a scanning multiwall spectrophotometer. All through the MTT test, the 96-well plates were kept away from direct light until crystals were completely solubilized. After 4 hours, a Spectra-Max M5 multi-plate reader was used to read the resulting purple solution at 560 nm for absorbance measurement. The amount of purple formazan product measured by the absorbance at 560 nm is directly proportional to the number of living cells in culture.

In this study, we ran the MTT assay multiple times until we recognized the toxic concentration and determined how long it took to show the toxic effect of H_2O_2 on the human brain vascular pericytes cells without killing these cells. First, we subcultured the cell in 96- well plate at 2 x 10^4 cell/200 µl media and incubated them for 48 hours to allow attachment and achieve a high confluence (70%). We tested the cytotoxicity in respect to concentration gradient and time course. We prepare stock H_2O_2 by dissolving 230 µl of 30% H_2O_2 in 10 ml of sterile water then prepare 1:30 dilution and read the absorbance at 215 nm three times and average them. We used the average absorbance and Beer –Lambert Law ($A=\varepsilon bc$), $c = (A/\varepsilon b)$*dilution factor & $C_1V_1= C_2V_2$ to calculate H_2O_2 concentration and prepare different concentration. In concentration gradient, the time of exposure was 1 hour. The medium was replaced with a 100 µl H_2O_2 concentration of: 0.1, 0.5, 1.0, 10 and 100 mM. At the same time, we had a control group containing cells without treatment. Additionally, we had a row with only MTT and media to

serve as background. Experiments were run three times each to ensure repeatability and decrease variation. We captured some images with the EVOS optical microscope to investigate cell morphologies then the plate was incubated in a humidified atmosphere with 5% CO2 at 37°C for one hour. We added 10 µl of MTT kit to each well with a repeating pipette. We then incubated the plate at 37°C for 4 hours in a humidified, 5% CO2 atmosphere. Then, we read the plate at 560 nm using Spectra-Max M5 multi-plate reader (Molecular Devices, Sunnyvale, CA) and recorded the absorbance. We repeated the test using H_2O_2 concentration of: 0.1, 0.2, 0.5 and 1.0 mM and control. To calculate the result, we subtracted the average of absorbance values of the culture medium background from all absorbance values of experimental values viability

$$\text{Viability \%} = \frac{experimental}{control} \text{ X } 100 \qquad (1)$$

In the time course we treated the cell with 200 µM H_2O_2 for different time periods: 4 hours, 3 hours, 2 hours, 1hour, 30 minutes and 0 minutes.

3.2.5 Lactate Dehydrogenase assay (LDH). It is colorimetric non radioactive assay, which quantitatively measures lactate dehydrogenase (LDH). LDH is a stable cytosolic enzyme that is released after cell lysis. LDH is present in all cells and it is rapidly released into the cell culture supernatant when the plasma membrane is damaged. The assay was performed in a 96-well plate. We used Roche cytotoxicity detection kit (LDH), which contains catalyst (Diaphorase/NAD^+ mixture) and Dye solution (INT and sodium lactate). The assay is dependent on the cleavage of a tetrazolium salt when LDH is present in the culture supernatant. We prepared a cell-free supernatant then incubated with INT to form colored formazan. The amount of color formed is proportional to the number of lysed cells. There are two enzymatic reactions

occurring here: first, LDH reduces NAD^+ to NAD + H^+ by oxidation of lactate to pyruvate. Second, enzymatic reaction diaphorase (the catalyst) transfers H/H^+ from NADH + H^+ to the tetrazolum salt INT. Visible wavelength absorbance data was collected using a Spectra-Max M5 multi-plate reader at 490 nm. As we did in the MTT assay, we also ran the LDH assay to recognized the most toxic concentration and how long it took to show the toxic effect of H_2O_2 on the human brain vascular pericytes cells. First, we subcultured the cell in a 96- well plate at $2x10^4$ cell/200 µl media and incubated for 48 hours to allow attachment and achieve a high confluence (70%). The medium was then replaced with the H_2O_2 to the desired test exposure period. Following this, we collected the cell-free culture supernatant and centrifuged them (spin at 1200 rpm for 5 min at 4º). We removed 100 µl supernatant and transferred it to another microplate and added100 µl/well reaction mixtures to each supernatant and incubated for 30 min. Experiments were run three times each to ensure repeatability and decrease variation. The sample in the plate contained culture media background, which is required to correct for contributions caused by phenol red and LDH activity that may be present in culture medium. The sample in the plate also contained positive control (cell with maximum LDH release) by adding lysis solution to the cell; negative control (cell with no LDH release), which is the cell without exposure to the toxic substance, and the experimental sample (treated cell with different H_2O_2 concentration).

In this study, we performed the test with concentration gradient and time course. We used H_2O_2 concentration of: 1, 0.5, 0.2, and 0 mM. To calculate the result, we subtracted the average of absorbance values of the Culture Medium background from all absorbance values of experimental, positive control and negative control, and then calculated the cytotoxicity with this equation.

$$\% \text{ cytotoxicity} = \frac{Experimental - Negative\ control}{Positive\ control - Negative\ conlrol} \text{ X } 100 \qquad (2)$$

In the time course we treated the cell with 200 µM H_2O_2 for different time periods: 4 hours, 3 hours, 2 hours, 1 hour, 30 minutes and 0 minutes.

3.2.6 Cellular morphology characterization. Cell morphology is an important sign of toxicity in cells. To find out the morphological changes on cell after performing cytotoxicity tasting, cell structure of treated cells is analyzed and compared to healthy untreated cells under an optical microscope. We measured the morphological changes after 1 hour of treatment the cells with 100 and 200 µM H_2O_2. Also we measured the morphological changes after treated the cells with 200 µM H_2O_2 at different time period(0 h,0.5 h, 1h, 2h,3h and 4h). Morphology is important, because cells will begin to deteriorate when the cellular structure is distorted or impaired. Signs of deterioration of cells include granular nucleus, formation of cytoplasmic vacuoles and cell detachment from surface. Measuring cell morphology deterioration is one way of measuring cell toxicity on cells.

3.3 Intracellular Proteins Expression Level

We tried to check the protein expression level, including F-actin, α-actin, PDGFR-β, tropomyosin and myosin. Intracellular Proteins expression level was examined by quantitative immunofluorescent microscopy. Proteins were labeled with specific primary antibodies and secondary antibodies, conjugated with different fluorescent probes.

3.3.1 Immunofluorescent staining of intracellular protein. The purpose of immunofluorescence staining is to detect the location and amount of specific proteins in cells. In this study, we detected F-actin using Actins green stain. We also detected α – Actin, PDGF-β, myosin and tropomyosin, using primary and secondary antibodies to label these proteins. Using

immunofluorescence, we can see when, where and how much protein is expressed in the cell after exposure to H_2O_2. Cell staining can be divided into four steps: cell preparation, fixation, application of antibody and evaluation. In cell preparation, the cell was attached to a coverslip to allow solid support and easy handling in subsequent procedures. Fixing and permeabilizing the cells ensures free access of the antibody to its antigen. Fixation immobilizes the antigens. Permeabilization permits access of antibodies to all cells and subcellular compartments. We fixed the cells with formaldehyde, which is a cross-linking reagent. Then, we incubated cells with the primary and secondary antibodies. Finally, the staining was evaluated using EVOS FL fluorescence microscopy.

First we autoclaved coverslips for 25 minutes and placed them in a 12-well plate coated with poly L-lysine. We then subcultured $3x10^4$ cells/well in1 ml of media and allowed them to adhere on coverslips and reach 50-60% confluent for 2 days to be able to get good images under the microscope. We then replaced the culture media with H_2O_2 (0, 100, 200 µM concentration) and left them for one hour. We used triplicate coverslips for each concentration. Next, we washed the coverslips twice with Phosphate Buffered Saline (PBS). We fixed the cells with 4% formaldehyde for 15 minutes in room temperature. Fixation is important to retain the shape and location of cellular protein. We washed three times with (PBS) for 5 minutes with shaking. Next, we added 1% Triton X-100 in PBS for 5 minutes. Triton X-100 is a permeabilization reagent that dissolves small holes in the cell membrane, giving antibodies access to cytoplasm and bind proteins. Next, we washed twice in PBS (5 minutes/ wash). We covered the cells with blocking solution. We used 5% goat normal serum in PBS. We incubated the samples for 1 hour in room temperature with gentle shaking. Next, we diluted the primary antibody in 1% blocking solution (1µl primary antibody for each 500 µl blocking solution). We incubated with sample overnight at

4ºc with gentle shaking. We washed three times in PBS (5 minutes/wash) with gentle shaking. We diluted the secondary antibody in 1% blocking solution (1µl primary antibody for each 1000 µl blocking solution). We incubated with sample for 1 hour with gentle shaking. We washed three times in PBS (5 minutes/ wash) with gentle shaking. We diluted Actins green solution 2 drops in 1 ml PBS. We incubated the Actins green with cells for 1 hour, and then washed three times in PBS (5 minutes/ wash) with gentle shaking. Next, we added one drop of anti-fade reagent on the slide then flipped the coverslip on top of it. We then put the coverslip in 4˚ overnight. We took pictures with an immunofluorescence microscope.

3.3.2 Primary antibodies. Antibodies are an important material used to detect the presence and the subcellular localization of an antigen in the cells. In our research, we used four different antibodies to detect the proteins inside the cells.

3.3.2.1 α–Actin (1A4). Is a mouse monclonal antibody used to detect smooth muscle α–actin of mouse, rat and human origin. Actin forms about 50% of total cellular protein. It detects the protein by western blotting, immunofluorescence and solid phase ELISA. The dilution used is different from test to test. Molecular weight of α-Actin: 43KDa.

3.3.2.2 PDGFR-β (958). Is a rabbit polyclonal antibody used to detect PDGF receptor type β in a mouse, rat and human origin. PDGF consists of 2 chains, A and B, which form three isoforms. These three isoforms bind with different affinities to two receptor types, PGDF-α and β. Platelet-derived growth factor receptor-β is a rabbit antibody raised against amino acid of PDGFR-β of human origin. PDGFR-β can bind to the B subunit of platelet-derived growth factor. It detects the protein by western blotting, immunofluorescence and solid phase ELISA. The dilution used is different from test to test. Molecular weight of PDGFR –β: 180-190KDa.

3.3.2.3 Tropomyosin (FL-2840). Is a rabbit polyclonal antibody used to detect Tropomyosin in the cell, which is a group of structural proteins binding actin filaments and functioning to modulate actin-myosin interaction and stabilize actin filament structure. It is used to detect 4 types of tropomyosin (α, β, γ,and 4) of mouse, rat and human origin by western blotting, immunofluorescence and solid phase ELISA. The dilution used is different from test to test. Molecular weight of tropomyosin: 31-47 KDa.

3.3.2.4 Myosin (FL-172). Is a rabbit polyclonal antibody raised against amino acids 1-172 used to detect myosin. Myosin is a protein that interacts with actin to generate the force of cellular movements. There are three classes of myosin: smooth muscle myosins, striated muscle myosins and non-muscle myosins. They regulate contraction in smooth muscle and non-muscle cells via phosphorylation by myosin light chain kinase (MLCK). The myosin antibody is used for detection of myosin regulatory light chains (Santa Cruz biotechnology, INC).

3.3.3 Secondary antibodies. They are fluorochrome-labeled secondary antibodies used to detect protein indirectly. In this experiment, we used rabbit anti mouse IGg and goat anti rabbit IGg.

3.3.4 Immunefluorescent microscopy. The EVOS Fl digital inverted microscope uses fluorescence to generate images. Antibodies are labeled with fluorochromes to allow visualizing the protein inside the cells. These labeled antibodies bind indirectly or directly to the antigen of interest. In the direct method, the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. But in the indirect method the antibody against the molecule of interest is unlabeled. It is called primary antibody and should be used with labeled secondary antibody.

3.4 Statistical Analysis

Statistical analysis was performed using Graphpad prism software. One-way ANOVA test (analysis of variance) was followed by Tukey test (all pairwise comparisons). Values are presented as means ± S.D. from three replicates for cytotoxicity testes and from 8 pictures to calculate the protein expreation intensity. Levels of significance and respective symbols are as follows: significance, $p \leq 0.05$, and no significance, ns.

CHAPTER 4

Results

4.1 Cytotoxicity Tests

We used hydrogen peroxide (H_2O_2) to mimic the ischemic condition. The idea is to introduce enough oxidative stress, which leads to pericytes activation but without killing the cells. To determine the optimal concentration of hydrogen peroxide for this study, pericytes were treated with different concentrations of hydrogen peroxide for different times. Cytotoxicity tests (including MTT and LDH) and cell morphology measurements were implemented. Tests were run in triplicate to ensure repeatability. Statistical analysis was performed using Graphpad prism software; the data are presented as means ± S.D.

4.1.1 Cell viability assay (MTT). In this study we ran the test with different concentration gradients and at different time courses.

4.1.1.1 Effect of hydrogen peroxide concentration on pericytes viability. When we exposed the cells to H_2O_2 concentration of 0.0, 0.1, 0.5,1,10, 100 mM for 1 hour, we found that the absorbance was highest at 0.1mM H_2O_2 compared to the untreated cell. The absorbance was lowest at 100mM H_2O_2. Absorbance level varied in the other concentrations. This indicates that the cell is more viable at 0.1 mM and less viable at 100mMcomparedto the untreated cells.
In figure 4 the values are presented as Means ± S.D from three samples. We observed that the cell loses its viability at 100 mM H_2O_2. The viability increases when H_2O_2 concentration decreases. The viability percentage is around 50 % at 1m M H_2O_2. This means that, above 1m M H_2O_2 is harmful to the cells. We decided to repeat the test with different concentration, ranging from 1to 0.1 mM. We chose H_2O_2 concentration of 0.0, 0.1, 0.2, 0.5, 1 mM and the control. Figure 5 illustrates the cell viability; the values are presented as Means ± S.D from three

samples. Comparing with the control, the figure shows that the cells were more viable at 0.1 mM H_2O_2 and less viable at 1 mM H_2O_2. Above 0.5 mM H_2O_2 is harmful to the cell. So, to be able to study the cell behavior under oxidative stress conditions, we should use H_2O_2concentration less than 0.5 mM.

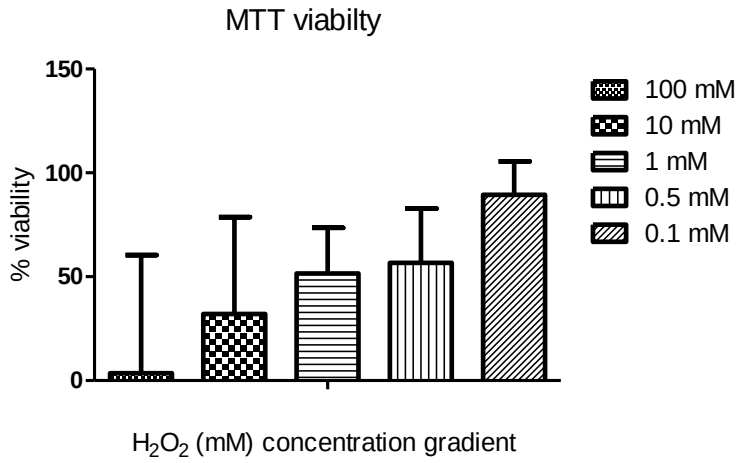


Figure 4. Effect of H_2O_2 concentration on pericytes viability; 0.1, 0.5, 1, 10 and100 mM. Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, p>0.05; n=3

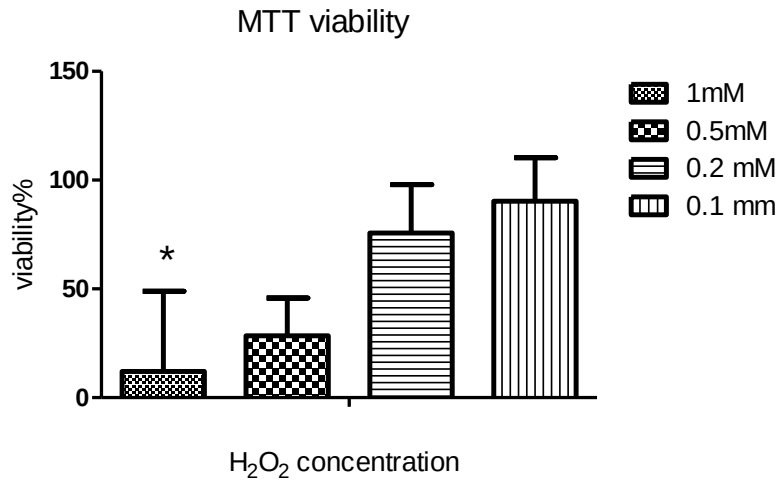


Figure 5. MTT viability assay with H_2O_2 concentration; 1, 0.5, 0.2 and 0.1 mM.

Statistical studies show that the P value is significant using one way ANOVA test followed by Tukey test (all pairwise comparisons). *, $p<0.05$ compared to 0.01mM. Values are Means ± S.D. n=3

4.1.1.2 Effect of time exposure to hydrogen peroxide on pericytes viability. To determine the time dependence of the cytotoxic potency of H_2O_2, we exposed the cell to 200 µM H_2O_2 for 4 h, 3h, 2 h, 1 hour, 30 minutes and 0 minutes. We determined cytotoxicity as a decrease in the cell viability compared to controls.

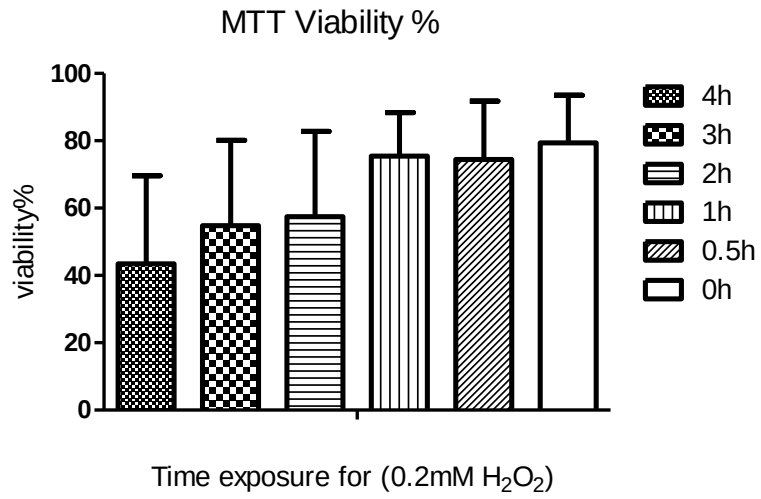


Figure 6. Effect of time exposure to hydrogen peroxide on pericytes viability.

Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, $p>0.05$; n=3

Figure 6 shows that there is a trend for difference, which looks like a time-dependent decrease in viability of pericytes, with increased time exposure to hydrogen peroxide. However, these levels did not reach formal significance. Statistical studies show that the P value is not significant.

4.1.2 Lactate dehydrogenase assay (LDH). We ran the LDH assay at diverse timeframes until we recognized the toxic concentration that causes activation of the cells without killing the cells. The various timeframes also enabled us to determine how long it takes to show

the toxic effect of H_2O_2 on the human brain vascular pericytes cells. We set two courses; concentration gradient and time course.

4.1.2.1. Effect of hydrogen peroxide concentration on pericytes with LDH. The cytotoxicity was assessed with the LDH assay at H_2O_2 concentration; 1, 0.5, 0.2 and 0 mM for 1 hour exposure. Figure 7 shows a trend for difference in cytotoxicity to pericytes with decreased hydrogen peroxide concentration but these levels did not reach formal significance. Statistical studies show that the P value is not significant.

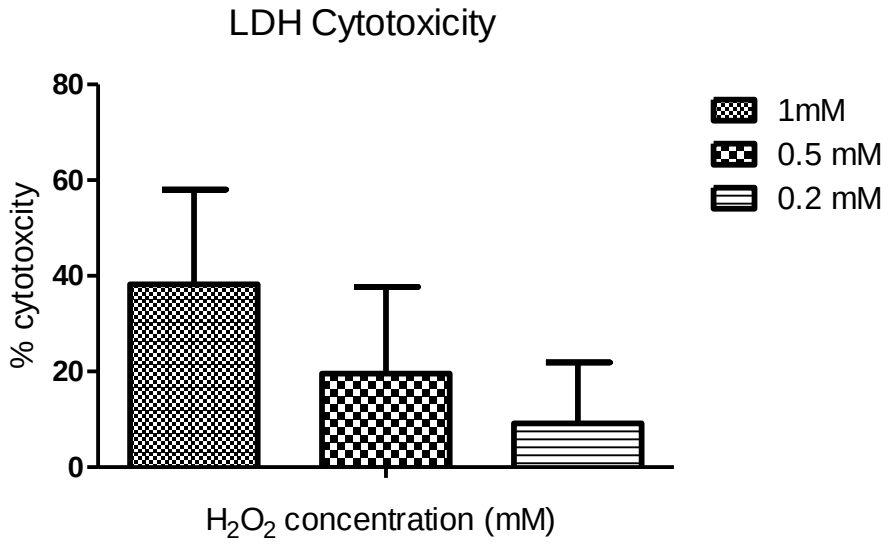


Figure 7. LDH cytotoxicity with H_2O_2 concentration; 1, 0.5 and 0.2mM H_2O_2.
Data are presented as mean ± S.D. from three experimental values one way ANOVA test with tukey's test showed that there is no significance deference , $p>0.05$.

4.1.2.2 Effect of time exposure to hydrogen peroxide on pericytes with LDH. As we did in the viability test, we ran the LDH test to determine the time-dependence of the cytotoxic potency of H_2O_2. We exposed the cell to 200 µM H_2O_2 for 4 h, 3h, 2 h, 1 h, 30 minutes and 0 minutes. The cytotoxicity is determined by the release of LDH into the culture media.

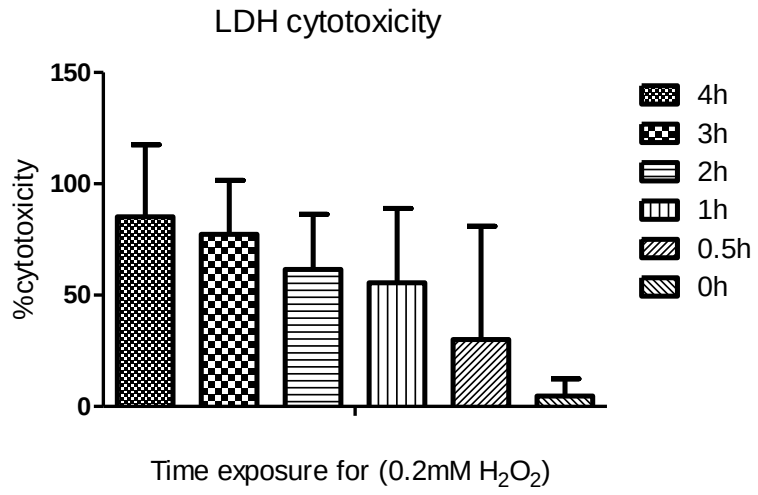


Figure 8. Time-dependent LDH cytotoxicity test.

Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, $p>0.05$; n=3

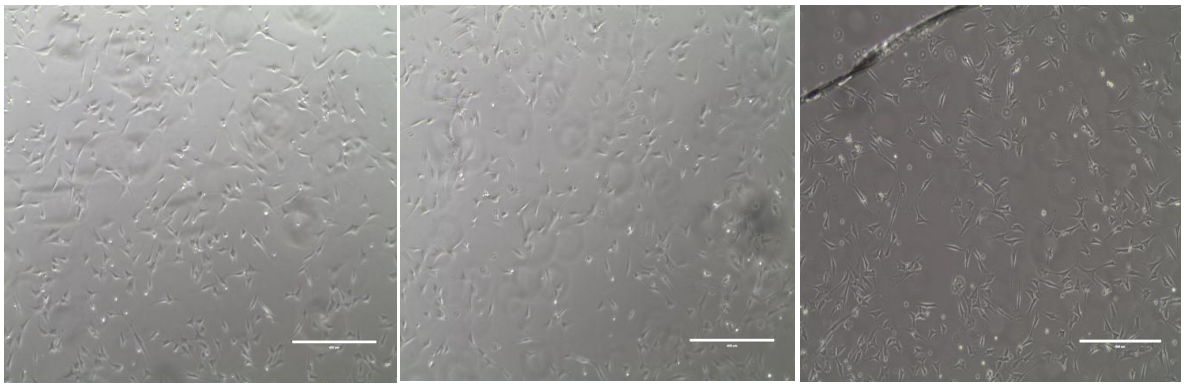
Figure 8 shows that there is a trend for cytotoxicity increases with longer time exposure for H_2O_2 but these levels did not reach formal significance. Statistical studies show the P value has no significant difference. At 4 h exposure, the H_2O_2 toxicity is around 80% and it is close to 50% at 1 h exposure.

Based on collected data, we chose to treat the pericytes with hydrogen peroxide at 100 µM and 200 µM for 1 h to produce oxidative stress condition similar to the hypoxic ischemic injury.

4.1.3 Characterization of cellular morphologies. Morphology was used to measure any changes in human brain vascular pericytes. Using the EVOS XL optical microscope, images were captured of each well in 96-well plate before running cytotoxicity testing.

4.1.3.1 Morphological changes with hydrogen peroxide concentration gradient. Figure 9 (a) shows the normal pericytes cell morphology, which are spindle-shaped and have a non-granular cytoplasm. After cellular exposure for hydrogen peroxide at 100 and 200 µM we noted

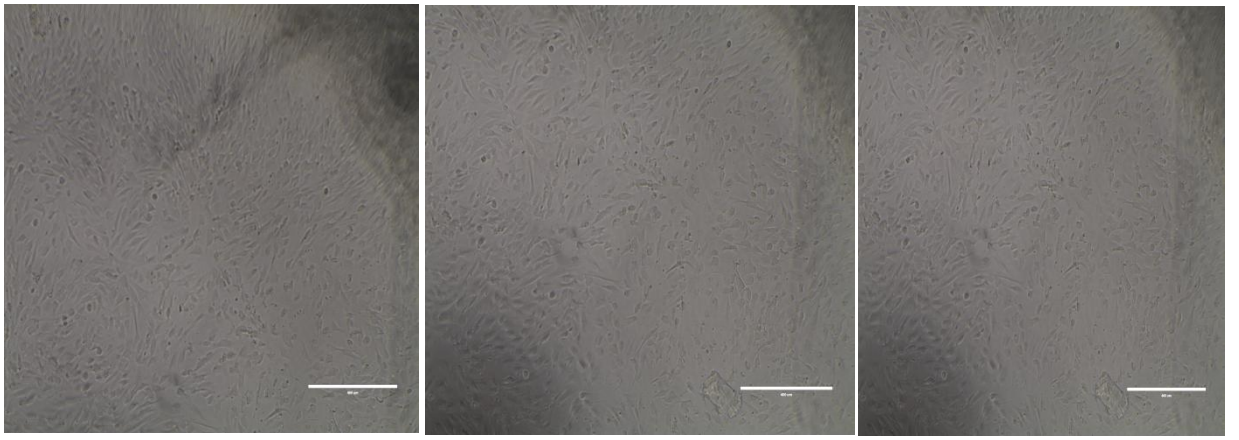
morphologic changes as cellular detachments from the surface, change of membrane integrity and appearance of cytoplasm. The morphological changes were more evident in figure 9 (c) where the cells were exposed to 200 µM H_2O_2 than in figure 9 (b) where the cells were exposed to 100 µM H_2O_2.



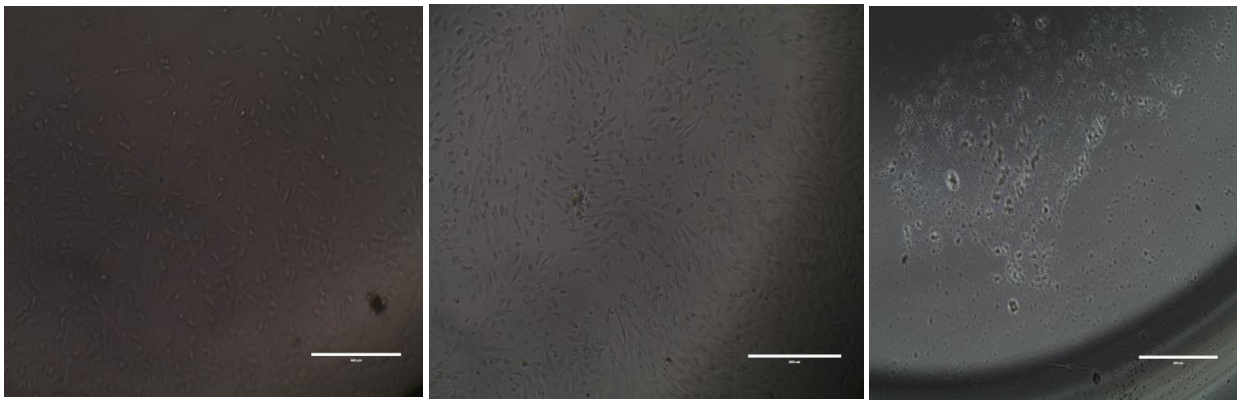
9 (a) HBVP 0 µM H_2O_2. 9 (b) HBVP 100 µM H_2O_2. 9 (c) HBVP 200 µM H_2O_2.

Figure 9. HBVP cells after exposed to H_2O_2 concentration of (0, 100,200 µM) for 1 h. Morphological changes were observed with 400X magnification micrograph.

4.1.3.2 Morphological changes with hydrogen peroxide time course. These pictures were captured for pericytes cells just after they were treated with 200 µM H_2O_2 and then at the time course of exposure before we ran the LDH test. Figure 10 (a) shows normal cell morphology. As time increases, the morphological changes increase. Figure 10 (f) shows the entire cells detached from the surface rounded and floated above the surface.



0h (b) 0.5h (c) 1h



(d) 2h (e) 3h (f) 4h

Figure 10. HBVP cells after exposed to 200 μM of H_2O_2 for different time courses. Morphological changes were observed with 400X magnification micrograph.

4.2 Intracellular protein expression

To evaluate the intracellular protein expression, the first step was staining the cells with actins green stain. This allowed us to visualize the F-actin microfilament with immunofluorescent microscopy. The second step was combining the actins green stain with the secondary antibodies, which were labeled with red immunofluorescent stain for specific proteins including; α-actin, myosin, tropomyosin, and PDGFR-β. After we captured the pictures, we

analyzed them to study the intensity and distribution pattern. We used image J software to measure the integrated density and calculate the intensity with prism program.

4.2.1 Effect of hydrogen peroxide on intracellular protein expression. The cells were stained with green fluorescent stain to visualize F- actin cytoskeleton and stained with red fluorescent stain to visualize α-actin, PDGFR-β, tropomyosin and myosin proteins in pericytes cells. We calculated intensity using 8-12 images, values presented as means ± S.D.

4.2.1.1 Effect of hydrogen peroxide on F-actin proptein expression. We measured the F-actin green stain intensity in the cell without exposure to hydrogen peroxide and after exposure to 100 µM and 200 µM for 1hour. The graph in figure 11 shows that the intensity of the F-actin green stains increases when the hydrogen peroxide concentration increases.

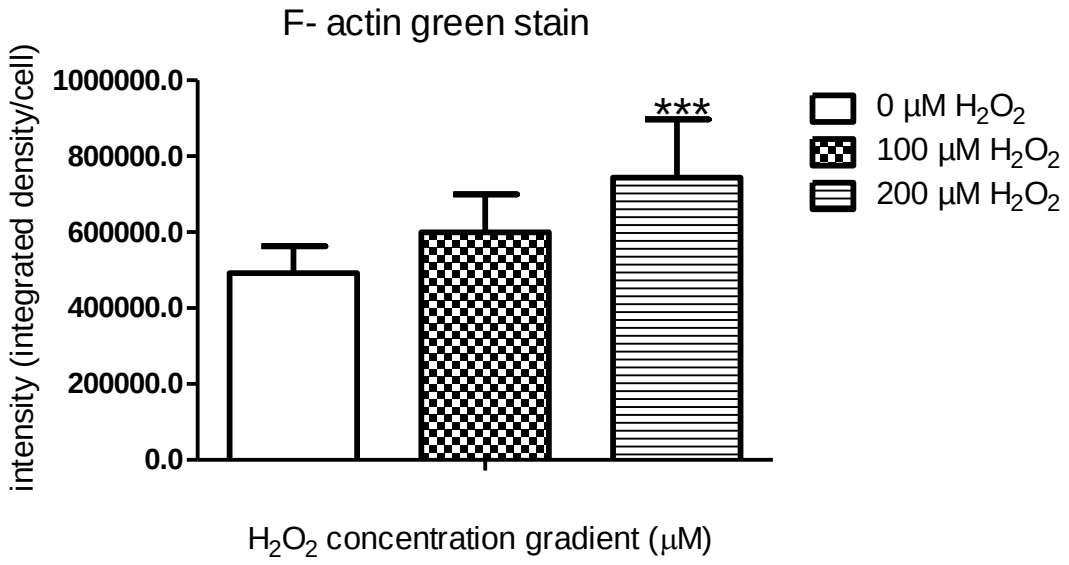


Figure 11. Fluorescence intensity of F-actin in hydrogen peroxide treated pericytes. Statistical studies show that the P value is significant. Values are Means ± S.D. ***, $p< 0.0001$; n=10 There is significant increase in fluorescent intensity of F- actin in hydrogen peroxide treated pericytes as compared with control.

To study the F-actin protein distribution pattern in cells, we applied immunofluorescent microscopy to visualize the F-actin at 3 levels of concentration of H_2O_2: Control, 100 µM, and 200 µM.

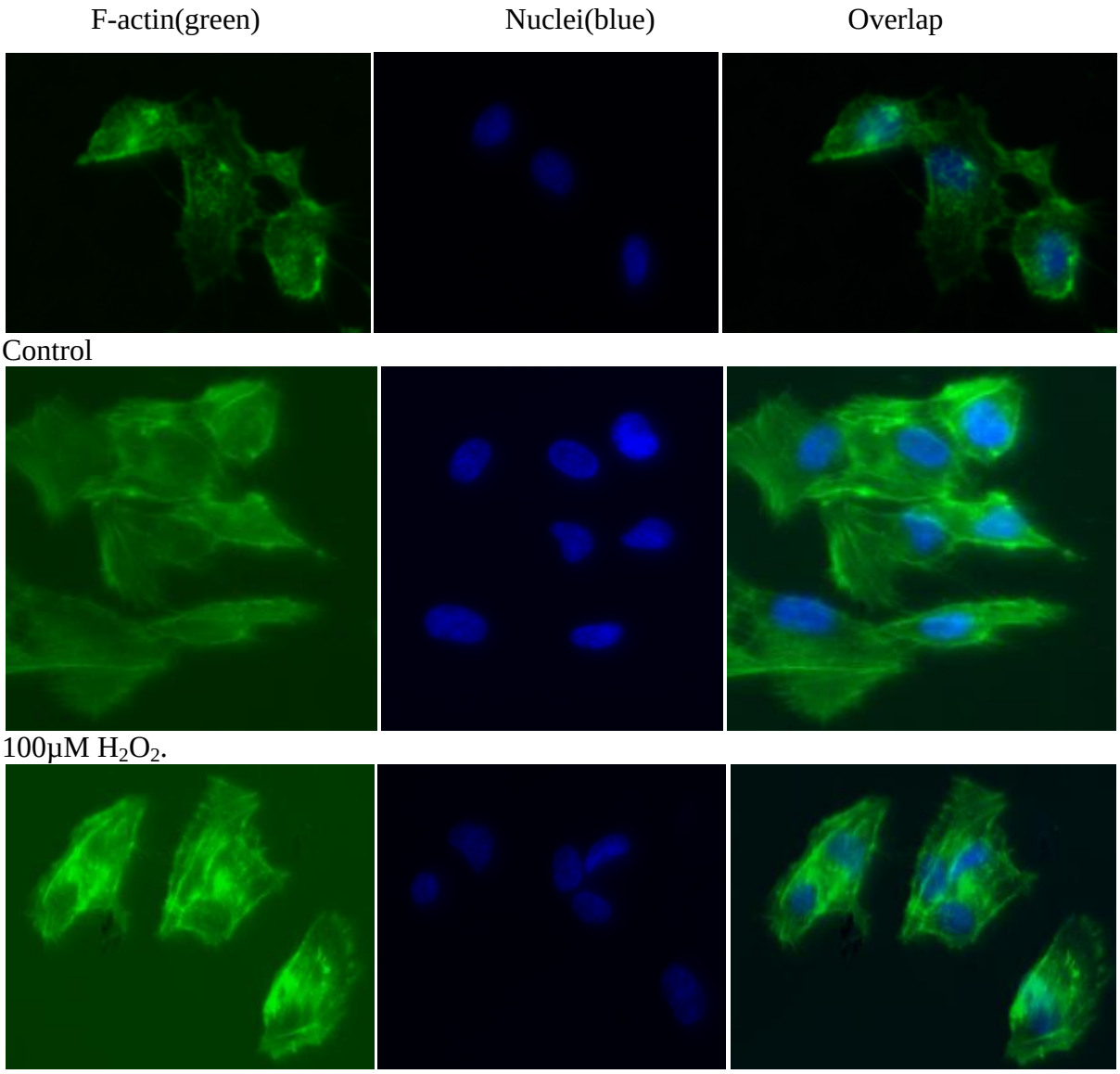


Figure 12. Effect of hydrogen peroxide on F-actin morphology and distributions.

400 X magnification micrograph shows the formation of cellular protrusion, and formation of nanotubes connecting the cells together when the cells treated with 100 µM H_2O_2 and 200 µM H_2O_2.

4.2.1.2 Effect of hydrogen peroxide on α-Actin protein expression. Figure 13 shows the red stain intensity of the α-actin antibody, there is a trend of difference when cells exposed to 100 µM H_2O_2 but this level does not reach formal significance. P value is ns $p > 0.05$

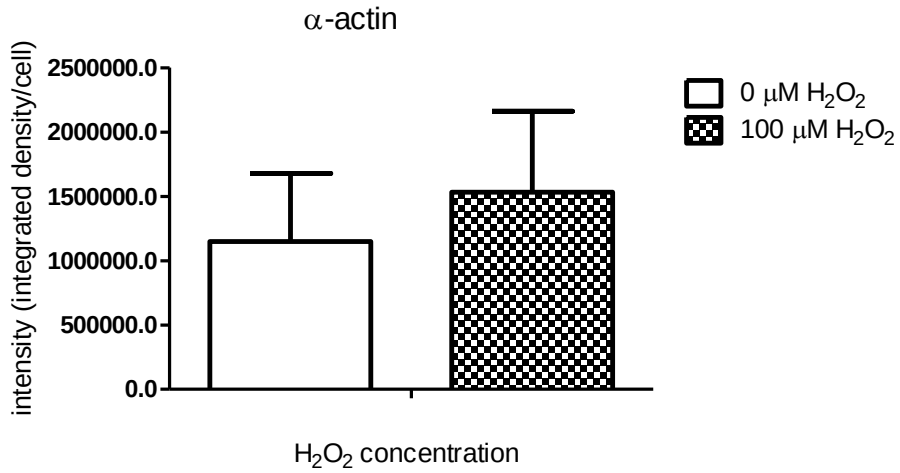


Figure 13. Fluorescence intensity of α-Actin in hydrogen peroxide treated pericytes. Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, $p>0.05$; $n=9$

There is no significant increase in fluorescent intensity of α Actin in hydrogen peroxide treated pericytes.

Pericytes were treated with 100 and 200 µM H_2O_2 to study the distribution pattern of α-Actin.

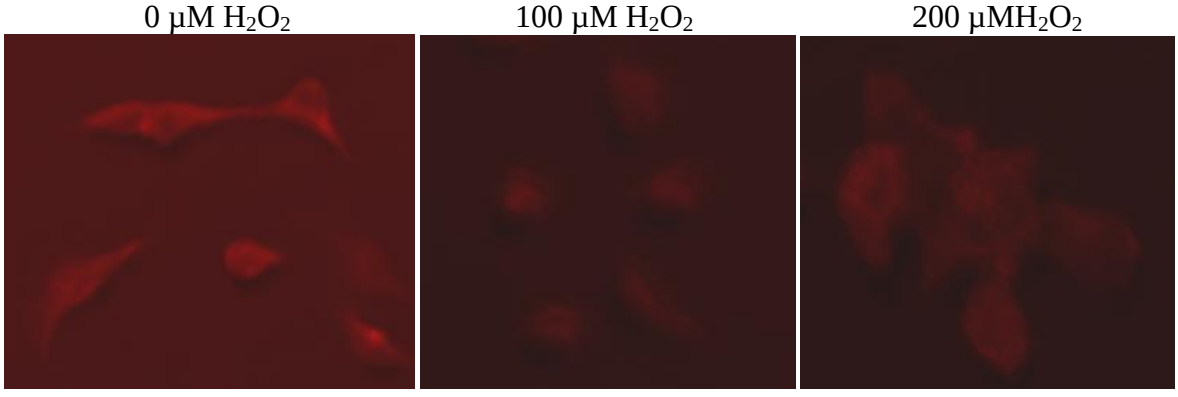


Figure 14. Effect of hydrogen peroxide on α-Actin morphology and distributions.

Distribution pattern of α-Actin was observed with 400X magnification micrograph.

4.2.1.3 Effect of hydrogen peroxide on PDGFR-β protein expression. We studied the PDGF-β protein intensity and distribution inside pericytes cells after treated with H_2O_2 for I h. Figure15 shows the red stain intensity of the PDGFR-β antibody, when cells exposed to 100μM H_2O_2.

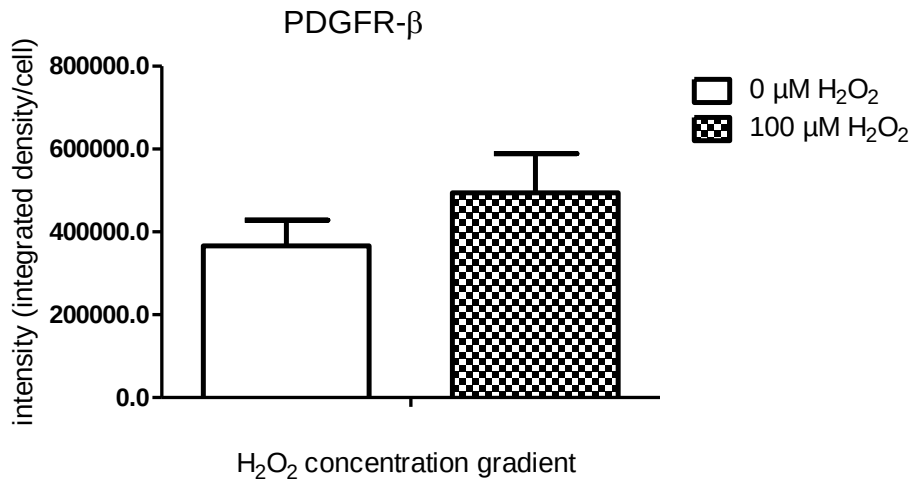


Figure 15. Fluorescence intensity of DGFR-β in hydrogen peroxide treated pericytes. Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, p>0.05; n=9

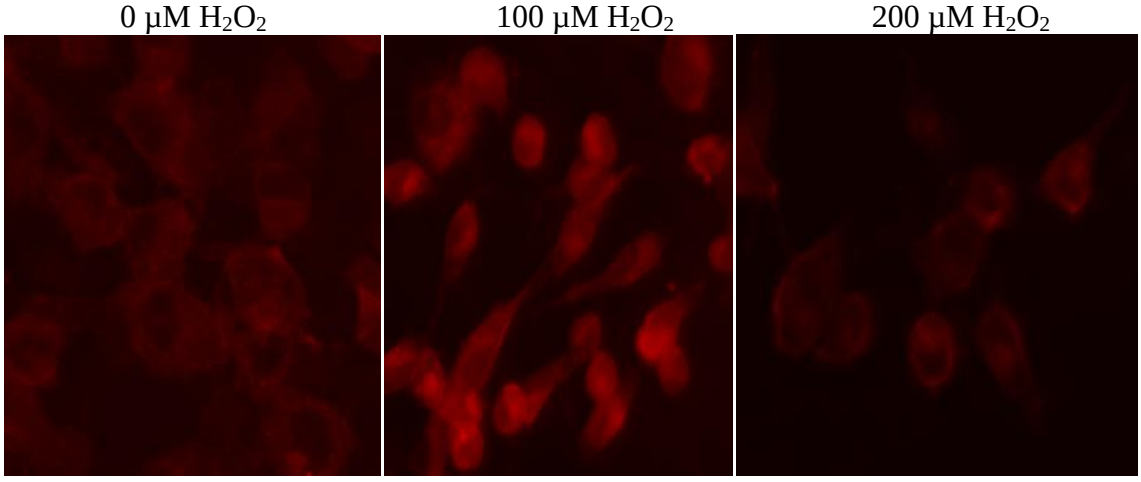


Figure 16. Effect of hydrogen peroxide on PDGFR-β morphology and distributions. Pericytes were treated with H_2O_2 for 1 h and subsequently immunostained for PDGFR-β. PDGFR-β distribution pattern was observed with 400X magnification micrograph.

4.2.1.4 Effect of hydrogen peroxide on myosin protein expression. The stain intensity in Figure17 shows the red stain intensity of the myosin antibody, which increases when cells exposed to 100 µM H_2O_2.

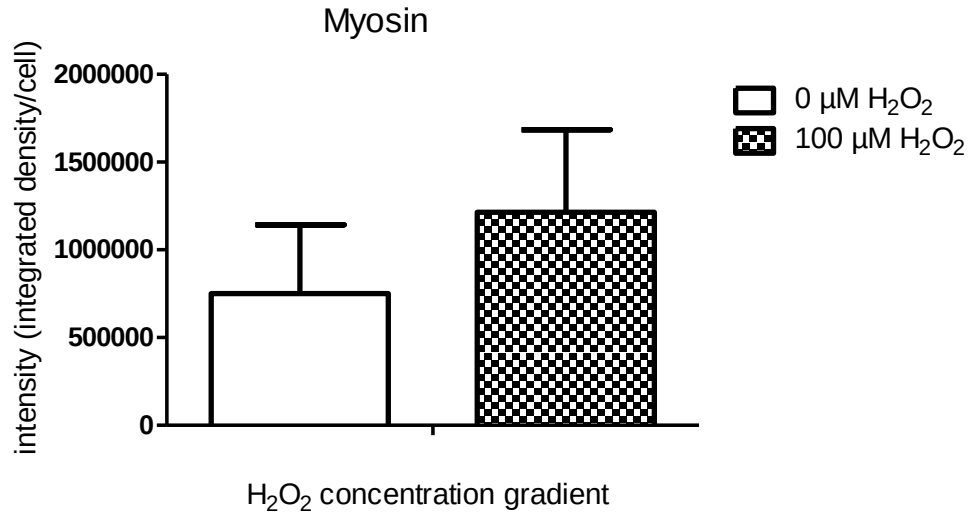


Figure 17. Fluorescence intensity of myosin in hydrogen peroxide treated pericytes. Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, p>0.05; n= 8

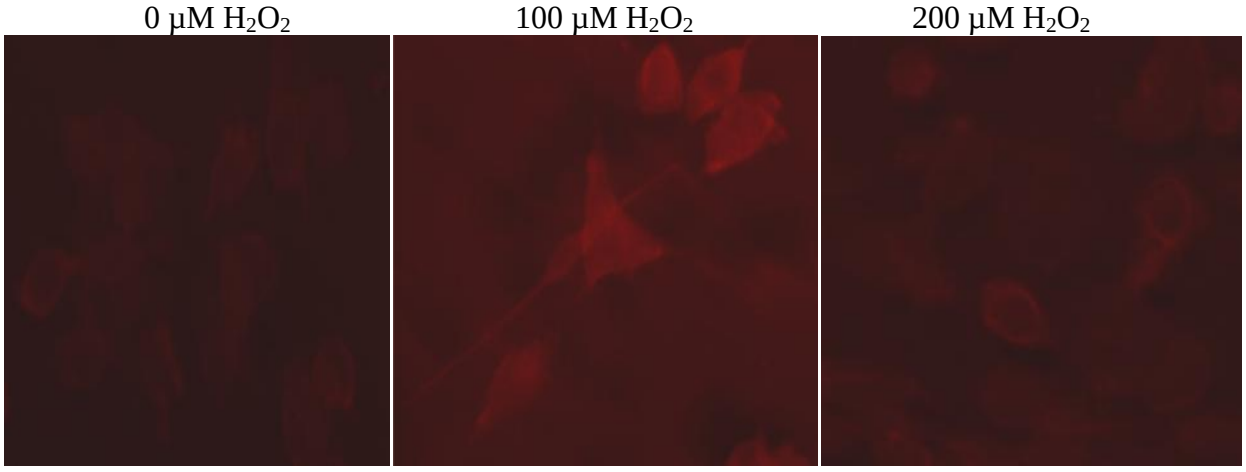


Figure 18. Effect of hydrogen peroxide on myosin morphology and distributions. Myosin distribution pattern was observed with 400X magnification micrograph.

4.2.1.5 Effect of hydrogen peroxide on tropomyosin protein expression. Figure 19 shows the red stain intensity of the tropomyosin antibody inside pericytes before they were treated with hydrogen peroxide and after exposed to 100 µM H_2O_2 for 1 h.

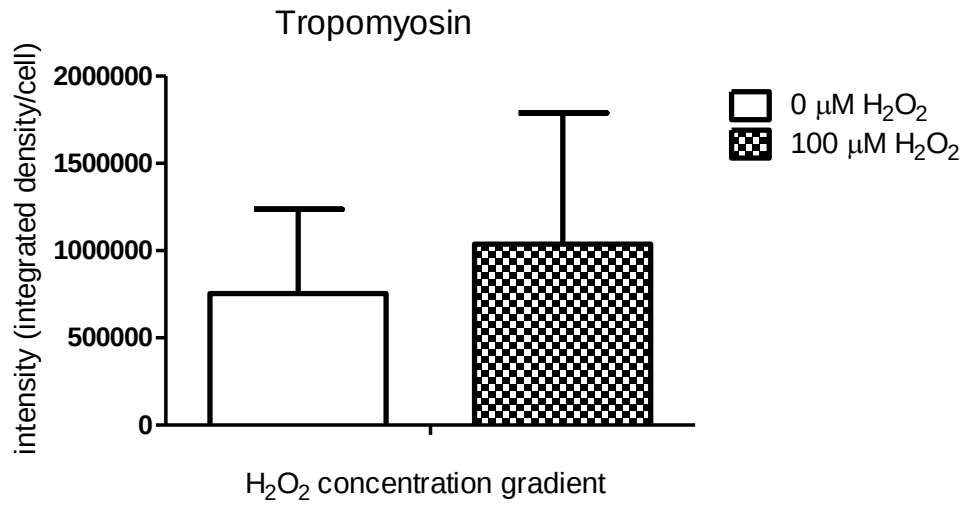


Figure 19. Fluorescence intensity of tropomyosin in hydrogen peroxide treated pericytes. Statistical studies show that the P value is not significant. Values are Means ± S.D. ns, $p>0.05$; n= 8

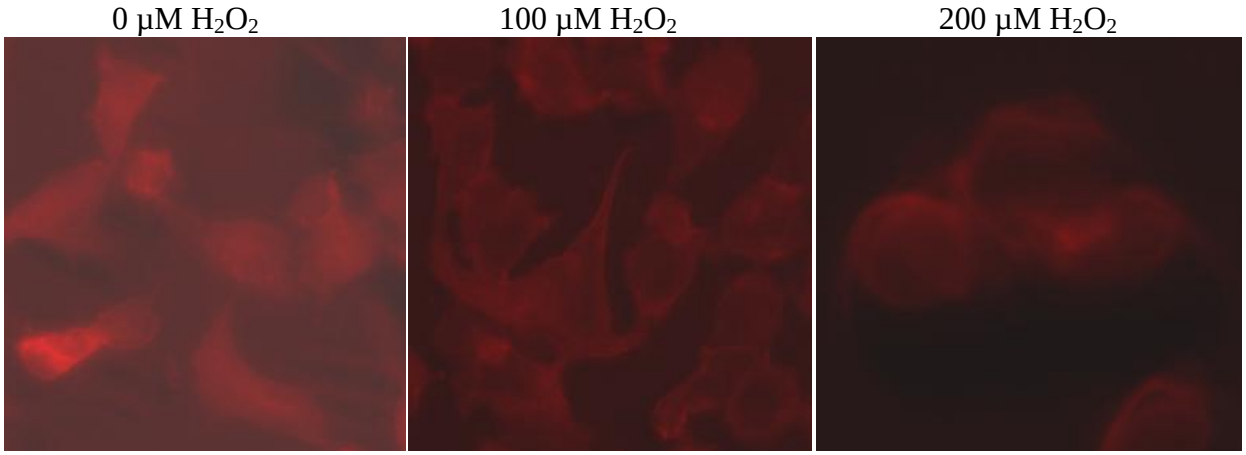


Figure 20. Effect of hydrogen peroxide on tropomyosin morphology and distributions. Tropomyosin distribution pattern was observed with 400X magnification micrograph.

Figure 12 shows that F-actin the stain intensity increased with increased concentration of hydrogen peroxide and the nucleus became granulated. We observed that H_2O_2 changed the distribution and position of F-actin and pushed it toward the cell membrane. This in turn, enhanced the formation of cellular protrusion, and formation of nanotubes connecting the cells together.

As figures (14, 16, 18 and 20) illustrate, the red stain for contractile protein distribution and pattern changes. Hydrogen peroxide enhances the protein movement toward the cell membrane. After treatment with H_2O_2 pericytes tend to form connection with each other. Reorganization of protein inside the cell was enhanced byH_2O_2 compared with non treated cells. Arrangement of protein around the cell membrane facilitates protein spread out and formation of cell protrusions and nanotubes between cells. This creates cell contraction and cell movement. This suggests that the contractile protein inside the cells is affected by oxidative stress.

Additionally, oxidative stress increases PDGFR-β expiration on pericytes. PDGFR-β is important in cell interaction between pericytes and endothelial cells. Endothelial cells secrets PDGF-B, which bind to PDGFR-β receptor in pericytes. This interaction is essential for presence pericytes in the vessels wall.

CHAPTER 5

Discussion and Future Research

5.1 Discussion

In this study, we examined the effect of oxidative stress on the protein expression, including F-actin, α-actin, PDGFR-β, tropomyosin, and myosin. We found that H_2O_2 increases the intensity of the protein in the cells. Regarding the distribution pattern of protein in pericytes cells, we observed that H_2O_2 changed this distribution. The protein changed position and moved toward the cell membrane, while sometimes forming cellular protrusion connecting cells together.

Our findings suggest that oxidative stress affect protein expression in HBVP cells, leading to changes in their distribution and intensity, which affects their function. This indicates that oxidative stress plays a role in cell contractility. Also, it plays a role in cell mobility and movement. Hamilton et al. (2010) reported that pericytes are contractile cells containing actin, myosin and tropomyosin microfilament. Yemisci et al. (2009) tested the effect of ischemic condition on pericytes on microvessels in the intact mouse brain. He reported that pericytes contraction was induced during ischemia. Our study on pericytes cell culture suggests that oxidative stress induces pericytes contraction. Dehouck et al. (1997) found that activation of pericytes endothelin-1 receptors lead to an elevation of intracellular calcium concentration, which lead to vasoconstriction of the cultured capillary pericytes. A study by Lindaure (2010) reported that vasoactive stimuli in an intact adult mouse brain leads to contraction of cortical capillary pericytes. Our study is consistent with the study done by Zhu et al. (2005) regarding F-

actin microfilament. We found that H_2O_2 increases F- actin expression and formation of cellular protrusions in pericytes cell. Zhu et al. (2005) reported the same result but in astrocyte cells.

There are different researchers who studied the effect of oxidative stress on astrocytes and they reported results similar to our results on pericytes. Qian et al. (2003) reported that oxidative stress causes cytoskeletal reorganization in astrocyte cells. Ramirez-Weber et al. (1999) described the effect of oxidative stress on the formation actin-enrriched protrusions. Regarding PDGFR-β, our study observed that H_2O_2 increases its expression inside the cell. PDGFR-β plays an important role in cell reaction between pericytes and endothelial cells. A study by Winkler et al. (2010) pointed to the role of PDGFR-β in pericytes recruitment to the vessel wall in the embryonic brain, regulating the development of the cerebral microcirculation and the blood-brain barrier (BBB). The author also showed that PDGFR-β is exclusively expressed in pericytes in the adult brain.

5.2 Conclusion

Pericytes are contractile cells, which are part of the neurovascular unit of the brain. They are located around blood microvessels in contact with endothelial cells. They play a role in the regulation of blood flow, vascular permeability, maintenance of microvessel wall integrity, and angiogenesis. The number of pericytes present in brain microvessels is higher than any other part of the body. To this date, the effect of oxidative stress on cerebrovascular pericytes cellular function has not been fully investigated.

In our study, we examined the effects of oxidative stress on cultured pericytes at passage 4 by exposing pericytes to H_2O_2, an oxidant commonly used to examine oxidative mechanisms mediating cell toxicity and death. We investigated how H_2O_2 concentration and exposure time affect cytotoxicity potency of H_2O_2 in vitro. Cytotoxicity tests measured the morphological effect

and viability of HBVP exposed to H_2O_2. We exposed pericytes to H_2O_2 at concentration ranging from 0.1 to 10 mM and exposure time ranging from 0 to 4 hours. Results from cytotoxicity tests suggest that when we exposed the human brain vascular pericytes cells to 100 µM and 200 µM for 1 hour, it showed the toxic effect of H_2O_2 on the human brain vascular pericytes cells without killing the cells. So, we chose to use 100 µM and 200 µM for 1 hour exposure to study how oxidative stress changes the proteins expression inside the cell.

5.3 Future Studies

The effect of oxidative stress on cerebrovascular pericytes cellular function has not been fully investigated. There is still limited information about pericytes biology under ischemic conditions. Future studies should illustrate in detail what and how oxidative stress may alter pericytes cellular function. These studies should determine how oxidative stress changes important cell membrane function receptors, i.e., Low-density lipoprotein receptor-related protein 1 (LRP1), the receptor for advanced gtycation end products (RAGE), and Platelet-derived growth factor receptor –β (PDGFR-β). Studies should investigate the changes of expression level of these receptors and their translocation inside the cell after exposure to oxidative stress.

Previous studies by Winkler et al. (2010) reported the role of PDGFR-β in mediating pericytes recruitment to the vessel wall and regulating the development of the cerebral microcirculation of BBB. So, future studies in PDGFR-β are needed to help explain the loss or impaired recruitment of pericytes to the outside endothelial wall in pathological conditions like AD and cerebral ischemia. Pericytes are contractile cells, which contain contractile protein like α-actin, myosine, and tropomyosin. These contractile proteins are responsible for pericytes

contraction. Future studies are, therefore, needed to provide information on how contractile protein and related cytoskeletal proteins are regulated or altered by oxidative stress.

References

Abramov, A. Y., Canevari, L., & Duchen, M. R. (2003). Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience, 23(12), 5088-5095.

Abramsson, A., Kurup, S., Busse, M., Yamada, S., Lindblom, P., Schallmeiner, E., . . . Gerhardt, H. (2007). Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. Genes Dev, 21(3), 316-331. doi: 10.1101/gad.398207

Al Ahmad, A., Gassmann, M., & Ogunshola, O. O. (2009). Maintaining blood–brain barrier integrity: Pericytes perform better than astrocytes during prolonged oxygen deprivation. Journal of Cellular Physiology, 218(3), 612-622. doi: 10.1002/jcp.21638

Armulik, Annika, Genove, Guillem, . . . Christer. (2010). Pericytes regulate the blood-brain barrier (Vol. 468). London, Royaume-University: Nature Publishing Group.

Attwell, D., Buchan, A. M., Charpak, S., Lauritzen, M., Macvicar, B. A., & Newman, E. A. (2010). Glial and neuronal control of brain blood flow. Nature, 468(7321), 232-243. doi: 10.1038/nature09613

Bandopadhyay, R., Orte, C., Lawrenson, J. G., Reid, A. R., De Silva, S., & Allt, G. (2001). Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. Journal of Neurocytology, 30(1), 35-44. doi: 10.1023/A:1011965307612

Barja, G. (2004). Free radicals and aging. Trends Neurosci, 27(10), 595-600. doi: 10.1016/j.tins.2004.07.005

Beckman, B., K., Ames, & N., B. (1998). The Free Radical Theory of Aging Matures (Vol. 78). Bethesda, MD, Etats-Unis: American Physiological Society.

Bell, R. D., Winkler, E. A., Sagare, A. P., Singh, I., LaRue, B., Deane, R., & Zlokovic, B. V. (2010). Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. Neuron, 68(3), 409-427. doi: http://dx.doi.org/10.1016/j.neuron.2010.09.043

Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., . . . Turnbull, D. M. (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nature genetics, 38(5), 515-517.

Bianca, V. D., Dusi, S., Bianchini, E., Dal Prà, I., & Rossi, F. (1999). beta-amyloid activates the O-2 forming NADPH oxidase in microglia, monocytes, and neutrophils. A possible inflammatory mechanism of neuronal damage in Alzheimer's disease. J Biol Chem, 274(22), 15493-15499.

Bonkowski, D., Katyshev, V., Balabanov, R. D., Borisov, A., & Dore-Duffy, P. (2011). The CNS microvascular pericyte: pericyte-astrocyte crosstalk in the regulation of tissue survival. Fluids Barriers CNS, 8(1), 8. doi: 10.1186/2045-8118-8-8

Brown, G. C., & Borutaite, V. (2004). Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. Biochimica et biophysica acta, 1658(1-2), 44-49.

Cogan, D. G., Toussaint, D., & Kuwabara, T. (1961). Retinal vascular patterns. IV. Diabetic retinopathy. Archives of ophthalmology, 66, 366-378.

Coyle, J. T., & Puttfarcken, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. Science, 262(5134), 689-695.

Crompton, M., Barksby, E., Johnson, N., & Capano, M. (2002). Mitochondrial intermembrane junctional complexes and their involvement in cell death. Biochimie, 84(2-3), 143-152.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W., & Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood, 105(5), 1950-1955. doi: 10.1182/blood-2004-05-1987

Dalfó, E., Portero-Otín, M., Ayala, V., Martínez, A., Pamplona, R., & Ferrer, I. (2005). Evidence of oxidative stress in the neocortex in incidental Lewy body disease. Journal of neuropathology and experimental neurology, 64(9), 816-830.

Dalkara, T., Gursoy-Ozdemir, Y., & Yemisci, M. (2011). Brain microvascular pericytes in health and disease. Acta Neuropathologica, 122(1), 1-9. doi: 10.1007/s00401-011-0847-6

Daneman, R., Zhou, L., Kebede, A. A., & Barres, B. A. (2010). Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature, 468(7323), 562-566.

Dehouck, M.-P., Vigne, P., . . . C. (1997). Endothelin-1 as a mediator of endothelial cell-pericyte interactions in bovine brain capillaries (Vol. 17). New York, NY, Etats-Unis: Nature Publishing Group.

del Zoppo, G. J., & Mabuchi, T. (2003). Cerebral microvessel responses to focal ischemia. J Cereb Blood Flow Metab, 23(8), 879-894. doi: 10.1097/01.wcb.0000078322.96027.78

Dore-Duffy, P., Katychev, A., Wang, X., & Van Buren, E. (2006). CNS microvascular pericytes exhibit multipotential stem cell activity. J Cereb Blood Flow Metab, 26(5), 613-624. doi: 10.1038/sj.jcbfm.9600272

Dore, S. E. (1923). On the Contractility and Nervous Supply of the Capillaries. *British Journal of Dermatology*, 35(11), 398-404. doi: 10.1111/j.1365-2133.1923.tb09069.x

Doulet, N., Donnadieu, E., Laran-Chich, M. P., Niedergang, F., Nassif, X., Couraud, P. O., & Bourdoulous, S. (2006). Neisseria meningitidis infection of human endothelial cells

interferes with leukocyte transmigration by preventing the formation of endothelial docking structures. J Cell Biol, 173(4), 627-637. doi: 10.1083/jcb.200507128

Ejaz, S., Chekarova, I., Ejaz, A., Sohail, A., & Lim, C. W. (2008). Importance of pericytes and mechanisms of pericyte loss during diabetic retinopathy. Diabetes, Obesity and Metabolism, 10(1), 53-63. doi: 10.1111/j.1463-1326.2007.00795.x

Fernández-Klett, F., Offenhauser, N., Dirnagl, U., Priller, J., & Lindauer, U. (2010). Pericytes in capillaries are contractile in vivo, but arterioles mediate functional hyperemia in the mouse brain. Proceedings of the National Academy of Sciences, 107(51), 22290-22295. doi: 10.1073/pnas.1011321108

Frank, R. N., Dutta, S., & Mancini, M. A. (1987a). Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. Invest Ophthalmol Vis Sci, 28(7), 1086-1091.

Frank, R. N., Dutta, S., & Mancini, M. A. (1987b). Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. Invest Ophthalmol Vis Sci, 28(7), 1086-1091.

Fridovich, I. (1999). Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Ann N Y Acad Sci, 893, 13-18.

Gandhi, S., & Abramov, A. Y. (2012). Mechanism of oxidative stress in neurodegeneration. Oxidative medicine and cellular longevity, 2012, 428010.

Geisler, S., Holmström, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J., & Springer, W. (2010). PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nature cell biology, 12(2), 119-131.

Geraldes, Pedro, Hiraoka-Yamamoto, Junko, . . . L., G. (2009). Activation of PKC and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy (Vol. 15). New York, NY, Etats-Unis: Nature Publishing Group.

Gerhardt, H., & Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. Cell and Tissue Research, 314(1), 15-23. doi: 10.1007/s00441-003-0745-x

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology, 40(8), 959-975.

Giorgio, V., Soriano, M. E., Basso, E., Bisetto, E., Lippe, G., Forte, M. A., & Bernardi, P. (2010). Cyclophilin D in mitochondrial pathophysiology. Biochimica et biophysica acta, 1797(6-7), 1113-1118.

Gonul, E., Duz, B., Kahraman, S., Kayali, H., Kubar, A., & Timurkaynak, E. (2002). Early pericyte response to brain hypoxia in cats: an ultrastructural study. Microvascular research, 64(1), 116-119.

Hamilton, N. B., Attwell, D., & Hall, C. N. (2010a). Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front Neuroenergetics, 2. doi: 10.3389/fnene.2010.00005

Hamilton, N. B., Attwell, D., & Hall, C. N. (2010). Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front Neuroenergetics, 2.

Harman, D. (1992). Free radical theory of aging. Mutat Res, 275(3-6), 257-266.

Hebert, L. E., Scherr, P. A., Bienias, J. L., Bennett, D. A., & Evans, D. A. (2003). Alzheimer disease in the us population: Prevalence estimates using the 2000 census. Archives of Neurology, 60(8), 1119-1122. doi: 10.1001/archneur.60.8.1119

Hellstorm, M., Kalen, M., . . . C. (1999). Role of PDGF-B and PDGFR-BETA in Recruitment of Vascular Smooth Muscle Cells and Pericytes During Embryonic Blood Vessel Formation in the Mouse (Vol. 126). Cambridge, ROYAUME-UNI: Company of Biologists.

Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H., & Betsholtz, C. (2001). Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol, 153(3), 543-553.

Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., . . . et al. (1995). Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. J Neurochem, 65(5), 2146-2156.

Herman, M., I., D'Amore, & A., P. (1985). Microvascular pericytes contain muscle and nonmuscle actins (Vol. 101). New York, NY, Etats-Unis: Rockefeller University Press.

Hirschi, K. K., & D'Amore, P. A. (1996). Pericytes in the microvasculature. Cardiovasc Res, 32(4), 687-698.

Kamouchi, M., Kitazono, T., Ago, T., Wakisaka, M., Kuroda, J., Nakamura, K., . . . Iida, M. (2007). Hydrogen peroxide-induced Ca2+ responses in CNS pericytes. Neuroscience Letters, 416(1), 12-16. doi: http://dx.doi.org/10.1016/j.neulet.2007.01.039

Kamouchi, M., Kitazono, T., Ago, T., Wakisaka, M., Ooboshi, H., Ibayashi, S., & Iida, M. (2004). Calcium influx pathways in rat CNS pericytes. Molecular Brain Research, 126(2), 114-120. doi: http://dx.doi.org/10.1016/j.molbrainres.2004.03.008

Krueger, M., & Bechmann, I. (2010). CNS pericytes: Concepts, misconceptions, and a way out. Glia, 58(1), 1-10. doi: 10.1002/glia.20898

Lai, C.-H., & Kuo, K.-H. (2005). The critical component to establish in vitro BBB model: Pericyte. Brain Research Reviews, 50(2), 258-265. doi: http://dx.doi.org/10.1016/j.brainresrev.2005.07.004

Lai, C. H., & Kuo, K. H. (2005). The critical component to establish in vitro BBB model: Pericyte. Brain Res Brain Res Rev, 50(2), 258-265. doi: 10.1016/j.brainresrev.2005.07.004

Lepoivre, M., Flaman, J. M., Bobe, P., Lemaire, G., & Henry, Y. (1994). Quenching of the tyrosyl free radical of ribonucleotide reductase by nitric oxide. Relationship to cytostasis induced in tumor cells by cytotoxic macrophages. J Biol Chem, 269(34), 21891-21897.

Lev, N., Ickowicz, D., Melamed, E., & Offen, D. (2008). Oxidative insults induce DJ-1 upregulation and redistribution: implications for neuroprotection. Neurotoxicology, 29(3), 397-405.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, 443(7113), 787-795.

Lindahl, P., Johansson, R., B., . . . C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice (Vol. 277). Washington, DC, Etats-Unis: American Association for the Advancement of Science.

Lotharius, J., & O'Malley, K. L. (2000). The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J Biol Chem, 275(49), 38581-38588.

Lovell, M. A., Ehmann, W. D., Butler, S. M., & Markesbery, W. R. (1995). Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. Neurology, 45(8), 1594-1601.

Marcus, D. L., Thomas, C., Rodriguez, C., Simberkoff, K., Tsai, J. S., Strafaci, J. A., & Freedman, M. L. (1998). Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. Exp Neurol, 150(1), 40-44. doi: 10.1006/exnr.1997.6750

Mathiisen, T. M., Lehre, K. P., Danbolt, N. C., & Ottersen, O. P. (2010). The perivascular astroglial sheath provides a complete covering of the brain microvessels: An electron microscopic 3D reconstruction. Glia, 58(9), 1094-1103. doi: 10.1002/glia.20990

Mattson, M. P. (2004). Metal-catalyzed disruption of membrane protein and lipid signaling in the pathogenesis of neurodegenerative disorders. Ann N Y Acad Sci, 1012, 37-50.

McCord, J. M. (2000). The evolution of free radicals and oxidative stress. Am J Med, 108(8), 652-659.

Mecocci, P., MacGarvey, U., & Beal, M. F. (1994). Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Annals of Neurology, 36(5), 747-751. doi: 10.1002/ana.410360510

Melgar, M. A., Rafols, J., Gloss, D., & Diaz, F. G. (2005). Postischemic reperfusion: ultrastructural blood-brain barrier and hemodynamic correlative changes in an awake model of transient forebrain ischemia. Neurosurgery, 56(3), 571-581.

Migliore, L., & Coppede, F. (2009). Environmental-induced oxidative stress in neurodegenerative disorders and aging. Mutat Res, 674(1-2), 73-84. doi: 10.1016/j.mrgentox.2008.09.013

Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, J., . . . Youle, R. J. (2010). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS biology, 8(1), e1000298.

Nelson, V. M., Dancik, C. M., Pan, W., Jiang, Z. G., Lebowitz, M. S., & Ghanbari, H. A. (2009). PAN-811 inhibits oxidative stress-induced cell death of human Alzheimer's disease-derived and age-matched olfactory neuroepithelial cells via suppression of intracellular reactive oxygen species. J Alzheimers Dis, 17(3), 611-619. doi: 10.3233/jad-2009-1078

Niwa, K., Porter, V. A., Kazama, K., Cornfield, D., Carlson, G. A., & Iadecola, C. (2001). A beta-peptides enhance vasoconstriction in cerebral circulation. American journal of physiology. Heart and circulatory physiology, 281(6), H2417-2424.

Palmer, A. M., & Burns, M. A. (1994). Selective increase in lipid peroxidation in the inferior temporal cortex in Alzheimer's disease. Brain Res, 645(1-2), 338-342.

Peppiatt, C. M., Howarth, C., Mobbs, P., & Attwell, D. (2006). Bidirectional control of CNS capillary diameter by pericytes. Nature, 443(7112), 700-704.

Perry, G., Castellani, R., Smith, M., Harris, P. R., Kubat, Z., Ghanbari, K., . . . Ghanbari, H. (2003). Oxidative damage in the olfactory system in Alzheimer's disease. Acta Neuropathologica, 106(6), 552-556. doi: 10.1007/s00401-003-0761-7

Poon, H. F., Calabrese, V., Scapagnini, G., & Butterfield, D. A. (2004). Free radicals and brain aging. Clin Geriatr Med, 20(2), 329-359. doi: 10.1016/j.cger.2004.02.005

Promega. (2009). CellTiter-Blue® Cell Viability Assay. Promega. Retrieved Part # TB317

Puro, D. G. (2007). Physiology and Pathobiology of the Pericyte-Containing Retinal Microvasculature: New Developments. Microcirculation, 14(1), 1-10. doi: 10.1080/10739680601072099

Qian, Y. Luo, J., Leonard, S. S., Harris, G. K., Millecchia, L., Flynn, D. C. and Shi, X. (2003). Hydrogen peroxide formation and actin filament reorganization by Cdc42 are essential for ethanol-induced in vitro angiogensis. *J. Biol. Chem*. 278.

Quaegebeur, A., Segura, I., & Carmeliet, P. (2010). Pericytes: blood-brain barrier safeguards against neurodegeneration? Neuron, 68(3), 321-323. doi: 10.1016/j.neuron.2010.10.024

Ramirez-Weber, F. A. and Kornberg, T. B. (1999). Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* 97.

Robb, S. J., & Connor, J. R. (1998). An in vitro model for analysis of oxidative death in primary mouse astrocytes. Brain Research, 788(1–2), 125-132. doi: http://dx.doi.org/10.1016/S0006-8993(97)01543-6

Rodrigo, R., Miranda, A., & Vergara, L. (2011). Modulation of endogenous antioxidant system by wine polyphenols in human disease. Clin Chim Acta, 412(5-6), 410-424. doi: 10.1016/j.cca.2010.11.034

Rouget, C. (1873). Mémoire sur le développement, la structure et les propriétés physiologique des cappilaires sanguins et lymphatiques.

Schenkel, A. R., Mamdouh, Z., & Muller, W. A. (2004). Locomotion of monocytes on endothelium is a critical step during extravasation. Nature immunology, 5(4), 393-400.

Shepro, D., & Morel, N. M. (1993a). Pericyte physiology. FASEB J, 7(11), 1031-1038.

Shepro, D., & Morel, N. M. (1993b). Pericyte physiology. FASEB J, 7(11), 1031-1038.

Sims, D. E. (1986). The pericyte--a review. Tissue Cell, 18(2), 153-174.

Sims, D. E. (2000). Diversity Within Pericytes. Clinical and Experimental Pharmacology and Physiology, 27(10), 842-846. doi: 10.1046/j.1440-1681.2000.03343.x

Smith, T. S., & Bennett, J. P. (1997). Mitochondrial toxins in models of neurodegenerative diseases. I: In vivo brain hydroxyl radical production during systemic MPTP treatment or following microdialysis infusion of methylpyridinium or azide ions. Brain Research, 765(2), 183-188.

Stadtman, E. R. (2004). Role of oxidant species in aging. Curr Med Chem, 11(9), 1105-1112.

Takahashi, A., Park, H. K., Melgar, M. A., Alcocer, L., Pinto, J., Lenzi, T., . . . Rafols, J. A. (1997). Cerebral cortex blood flow and vascular smooth muscle contractility in a rat model of ischemia: a correlative laser Doppler flowmetric and scanning electron microscopic study. Acta Neuropathologica, 93(4), 354-368.

Tallquist, M. D., French, W. J., & Soriano, P. (2003). Additive effects of PDGF receptor beta signaling pathways in vascular smooth muscle cell development. PLoS biology, 1(2), E52.

Thomas, T., Thomas, G., McLendon, C., Sutton, T., & Mullan, M. (1996). beta-Amyloid-mediated vasoactivity and vascular endothelial damage. Nature, 380(6570), 168-171.

Thomas, W. E. (1999). Brain macrophages: on the role of pericytes and perivascular cells. Brain Res Brain Res Rev, 31(1), 42-57.

Vives-Bauza, C., Zhou, C., Huang, Y., Cui, M., de Vries, R. L., Kim, J., . . . Przedborski, S. (2010). PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proceedings of the National Academy of Sciences of the United States of America, 107(1), 378-383.

Webb, R. C. (2003). Smooth muscle contraction and relaxation. Advances in physiology education, 27(1-4), 201-206.

Weller, R. O., Subash, M., Preston, S. D., Mazanti, I., & Carare, R. O. (2008). Perivascular drainage of amyloid-beta peptides from the brain and its failure in cerebral amyloid angiopathy and Alzheimer's disease. Brain pathology (Zurich, Switzerland), 18(2), 253-266.

Wilhelmus, M. M., Otte-Höller, I., van Triel, J. J., Veerhuis, R., Maat-Schieman, M. L., Bu, G., . . . Verbeek, M. M. (2007). Lipoprotein receptor-related protein-1 mediates amyloid-beta-mediated cell death of cerebrovascular cells. The American journal of pathology, 171(6), 1989-1999.

Yang, L., Froio, R. M., Sciuto, T. E., Dvorak, A. M., Alon, R., & Luscinskas, F. W. (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood, 106(2), 584-592.

Yemisci, M., Gursoy-Ozdemir, Y., Vural, A., Can, A., Topalkara, K., & Dalkara, T. (2009). Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. Nat Med, 15(9), 1031-1037. doi: 10.1038/nm.2022

Zhang, Y., Schauer, J. J., Shafer, M. M., Hannigan, M. P., & Dutton, S. J. (2008). Source apportionment of in vitro reactive oxygen species bioassay activity from atmospheric particulate matter. Environ Sci Technol, 42(19), 7502-7509.