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Examining the protective effects of sesamol on oxidative stress associated blood -brain barrier dysfunction in streptozotocin-induced diabetic rats

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Examining the protective effects of sesamol on oxidative stress associated blood-brain barrier dysfunction in streptozotocin-induced diabetic rats

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**Dissertation submitted to the
School of Pharmacy
at West Virginia University
in fulfillment of the requirements for the degree of**

Doctor of Philosophy

in

Pharmaceutical and Pharmacological Sciences

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ABSTRACT

Examining the protective effects of sesamol on oxidative stress associated blood-brain barrier dysfunction in streptozotocin-induced diabetic rats

Reyna VanGilder

Many studies point to vascular dysfunction as an underlying cause for the increased incidence of cognitive dysfunction and risk for development of Alzheimer's disease during diabetes. Vascular dysfunction is not an uncommon occurrence in patients with diabetes and microvascular dysfunction commonly leads to clinical complications such as blindness, peripheral neuropathy, and kidney failure. Microangiopathies of the retina, kidney, and peripheral nerves have been well-characterized; however, the effects of diabetes on blood-brain barrier (BBB) function have been understudied.

Pathophysiological changes defining microvascular dysfunction include basement membrane thickening, cytoskeleton rearrangement, and increased paracellular leakage. Increased paracellular leakage of the BBB suggests a functional break down of the tight junction. To investigate changes in functional integrity, we used three different sized vascular space markers [sucrose (342 Da), inulin (5000 Da), and evans blue (68,000 Da)] to measure time-dependant paracellular permeability changes. Our findings revealed that the smallest vascular space marker (sucrose) showed subtle region-specific permeability changes that may represent an altered neuronal microenvironment. Previously published clinical data coincides with these region-specific changes observed in the hippocampus, cortex and midbrain. Patients with diabetes have a higher incidence of midbrain-related lacunar infarcts and cognitive deficiencies can be correlated to areas like the hippocampus and cortex.

Sesamol, a natural antioxidant, has been shown to improve cognitive function in STZ-induced diabetic rats. Furthermore, microangiopathy studies show that oxidative stress plays a major role in microvascular dysfunction; therefore, we investigated if oxidative-stress contributed to BBB permeability. Rats were randomly divided into four treatment groups (CON- control; STZ- STZ-induced diabetes; CON+S- control+sesamol; STZ+S- STZ-induced diabetes+sesamol). Functional and structural BBB changes were measured by *in situ* brain perfusion with sucrose and tight junction expression was assessed by real time RT-PCR and western blot analyses. Oxidative stress markers were visualized by fluorescent confocal microscopy and assayed by spectrophotometric analyses. Results demonstrated that STZ+S rats showed increased tight junction protein expression and decreased permeability as compared to STZ treated rats. Furthermore, STZ+S treated rats show increased antioxidant enzyme activity and decreased markers of oxidative stress in the brain. In conclusion, this study showed that sesamol treatment enhanced antioxidant capacity of the diabetic brain and led to decreased perturbation of oxidative stress-induced changes in BBB structure and function.

Next, we investigated the antioxidant mechanism for sesamol and oxidative mechanisms that may contribute to enhanced BBB permeability. The chemical properties of sesamol permit passage through the BBB and suggest that Fenton-induced lipid peroxidation can be inhibited. The brain, possessing iron stores and high levels of polyunsaturated fatty acids, may be vulnerable to Fenton-induced lipid peroxidation under pro-oxidant conditions during diabetes.

Spectrophotometric assays were used to assess ferrous iron levels, hydrogen peroxide production, and lipid peroxidation in the brain. Furthermore, oxidative stress influences vascular remodeling and aberrant neovascularization of blood-retinal barrier (BRB) during diabetes. Because the BRB and BBB possess a similar structure and function, we examined whether similar pathophysiological changes occurred in the brain and if sesamol treatment influenced pathological changes. Gel zymography and real time RT-PCR were used to assess these parameters. Sesamol treatment reduced lipid peroxidation and enhanced mitochondrial superoxide dismutase (SOD) activity. Sesamol-related lignans can upregulate lipolytic enzymes, thus, sesamol may have exert similar effects. Elevated PDGF transcription in the STZ group was attenuated in the STZ+S group. PDGF plays a role in tight junction rearrangement and neovascularization in diabetic retinopathy, thus demonstrating neovascularizing factors may influence BBB integrity. This study suggests that sesamol may be beneficial as an adjuvant therapy for minimizing lipid peroxidative damage during diabetes.

The present results suggest that oxidative stress is a key factor promoting BBB dysfunction during STZ-induced diabetes and that sesamol or sesamol-related compounds might be beneficial adjuvant therapies for minimizing oxidative damage to the cerebral endothelium. Understanding the oxidative mechanisms contributing to BBB permeability may elucidate novel pharmacological targets for maintaining BBB function and promoting neuron survival. To accomplish this, more studies are needed to understand the signaling pathways connecting BBB integrity and supporting cells (e.g. astrocytes, microglia, pericytes).

Dedicated to

My Parents

Ron and Renee VanGilder

My support system

Ryan Simonton

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LIST OF ABBREVIATIONS

Abbreviation	Word
AD	Alzheimer's disease
AGE	Advanced glycation end-product
ATP	adenosine triphosphate
BBB	blood-brain barrier
CAT	catalase
CBF	cerebral blood flow
CNS	central nervous system
CON	control
DAG	diacylglycerol
DCCT	Diabetes Control and Complication Trial
DHE	dihydroethidium
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde dehydrogenase
GLUT	glucose transporter
GPx	glutathione peroxidase
GSH	glutathione
HbA1c	glycated hemoglobin
HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
i.p.	intraperitoneally
MDR	multidrug resistant protein
MMP	matrix metalloproteinase
MOA	monoamine oxidase

MRI	magnetic resonance imaging
NADPH	nicotinamide adenine dinucleotide phosphate
NFKB	nuclear factor kappa-light-chain enhancer of activated B cells
NO-	nitric oxide
O	superoxide anion
ONNO	peroxynitrite
PCR	polymerase chain reaction
PGP	p-glycoprotein
PKC	protein kinase c
PVDF	polyvinylidene difluoride
RAGE	receptor for advanced glycation end products
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
Trx	thiodoxin reductase
ZO-1	zonula occludens

CHAPTER ONE

Literature Review

1.1 Introduction

Diabetes affects more than 23.6 million people in the United States and nearly a quarter of these individuals are undiagnosed. Complications associated with poorly managed blood glucose or undiagnosed diabetes lead to several long term health complications. Over 65% of diabetes-related morbidity and mortality is related to cardiovascular and cerebrovascular diseases (Barrett-Connor and Khaw, 1988). The increased morbidity and mortality associated with diabetes is correlated with progressive dysfunction of the endothelium and associated alterations in hemodynamics leading to angiopathy of both large (macroangiopathy) and small (microangiopathy) blood vessels.

Microangiopathy is a primary factor in the development and progression of disabilities most commonly associated with diabetes, including blindness, kidney failure, and peripheral neuropathies (Schrijvers et al., 2004;Sima et al., 2008;Otero-Siliceo and Ruano-Calderon, 2003). Microangiopathy is clinically characterized by basement membrane thickening, cytoskeletal rearrangement, and increased paracellular leakage (Hill and Williams, 2004;Idris et al., 2004;Yu et al., 2005). Extensive research has been conducted on microangiopathies in a number of tissues, including kidney, peripheral nerves, retina, heart, and skeletal muscle (Hill and Williams, 2004;Basile et al., 2004;Pricci et al., 2003;Salmi et al., 2002;Gustafsson and Kraus, 2001). These studies have revealed that prolonged hyperglycemia, hypertension, dyslipidemia, insulin resistance and increased oxidative stress are important factors contributing to altered endothelial cell function (Liu et al., 2004;Megherbi et al., 2003;Osicka et al., 2003;Baird et al., 2002;Lee et al., 2002;Colwell, 2000).

Hyperglycemia-induced oxidative-stress mechanisms contribute to microvascular alterations of the kidney and retina. Furthermore, increased oxidative stress (Olesen, 1987) or decreased antioxidant enzyme activity (Agarwal and Shukla, 1999) directly correlates to altered cerebromicrovascular function. However, the role of hyperglycemia-induced oxidative stress with regard to cerebral microvascular dysfunction has been understudied. One possible explanation for this gap in knowledge is that vascular dysfunction in other tissues leads to observable changes that have a long-standing association with diabetes. Meanwhile, changes to central nervous system function are more subtle and worsen with time. Furthermore, due to the unique phenotype of the BBB (e.g. tight junctions and lack of fenestrations), the effects of diabetes on the cerebromicrovasculature are different from other microvascular beds and barrier systems, such as seen at the retina and peripheral nerves. However, recent clinical evidence does suggest that diabetes-induced changes in the BBB lead to increased incidences of vascular dementia, ventricular hypertrophy, lacunar infarcts, hemorrhage, and may be a predisposing factor for Alzheimer's disease (Ristow, 2004). Thus, determining the pathophysiological role of diabetes-induced oxidative stress on BBB function and structure will give insight toward understanding the increased susceptibility to cerebrovascular diseases often seen in individuals with diabetes and altered neuronal function.

1.2 Diabetes and Impaired Cognition

The association between diabetes and reduced cognition (Miles and Root,) has been acknowledged since the discovery of insulin and several studies demonstrated that people suffering from type 1 (T1D) and type 2 (T2D) diabetes have an increased risk of cognitive impairment (Bruce et al., 2008;Luchsinger et al., 2007;van et al., 2007). Yet, the American Diabetes Association does not currently recognize cognitive dysfunction as a diabetes-associated complication. With modern medicine better able to treat diabetes and prolong the lives of patients, the incidence of diabetes-related dementia will likely grow to become an important health concern.

The pathophysiology of diabetes in the central nervous system has not been well-characterized. However, it is surprising that the association between cerebral microangiopathy and increased risk of dementia has received little attention with microvascular dysfunction being a common diabetes-associated complication. Like microvascular dysfunction, cognitive impairment has been attributed to risk factors such as chronic hyperglycemia, increased oxidative stress, advanced cardiovascular disease, repeated hypoglycemic episodes, alterations of insulin function in the brain, and age-related changes in metabolism (Dahle et al., 2009;Helzner et al., 2009;Cukierman-Yaffe et al., 2009).

Two risk factors correlating with impaired cognition include poor blood glucose control and pre-existing microvascular complications. The Diabetes Control and Complication Trial (DCCT), an 18 year follow-up study, revealed a correlation between worsened cognitive function and poor long-term blood glucose control, measured by glycated hemoglobin (HbA1c) levels. T1D patients with normal blood glucose levels (HbA1c <7.4%) performed significantly better on

tests of motor speed and psychomotor efficiency when compared to subjects experiencing hyperglycemia (HbA1c >8.8) (Takeuchi et al., 2000; Jacobson et al., 2007). Additionally, acute hyperglycemia has been associated with slowing of all cognitive function (Cox et al., 2005), loss of focus (Rovet and Alvarez, 1997), and impaired working memory in patients with T1D and T2D (McAulay et al., 2006). In a meta-analysis study, the presence of diabetic complications was associated with worse cognitive function in T1DM (Brands et al., 2005). Difficulties with information processing speed, attention, and concentration were linked to the presence of retinopathy (Ferguson et al., 2003). In another study, the occurrence of distal symmetrical polyneuropathy was related to worse cognitive functions on most domains except for memory (Ryan et al., 1993). Pinpointing specific cognitive deficits has been challenging due to differences in methodologies, cohort samples, and other confounding factors. Regardless, poor blood glucose control and previous microvascular complications appear to be indicators of cognitive dysfunction.

Prevalence of cognitive dysfunction seen in people with either T1D or T2D is at least two-fold higher as compared to both normal blood glucose and impaired fasting blood glucose individuals (Gregg et al., 2000; Allen et al., 2004; Fuh et al., 2007; Haan et al., 1999). The impaired blood glucose tolerance associated with T2D has been associated with early cognitive deficits that worsen with age and vascular pathology (Yaffe et al., 2009). People with diabetes have increased risk for developing cognitive deficits as they age and are at increased risk for future dementia (Jellinger, 2008). Pathological changes such as grey matter atrophy (Yavuz et al., 2007; Biessels et al., 2006) and advanced glycation end-product (AGE) accumulation (Whitmer, 2007; Sato et al., 2006) are commonly seen in elderly people with diabetes or Alzheimer's disease as compared to aged-matched non-diseased elderly patients. Post-mortem

studies of patients with diabetes and dementia reveal both microvascular lesions and extensive amyloid plaque loads, thus suggesting diabetes is a risk factor for vascular-associated dementia and Alzheimer's disease (AD) (Biessels et al., 2002).

Hyperglycemia may play a major role in the progression of diabetes-associated dementia and Alzheimer's disease by altering both metabolic and vascular function in the brain (Pasquier et al., 2006). In addition, alterations in insulin secretion and insulin resistance, both systemically and centrally, play an important role in cognitive function and neurodegeneration (Yaffe et al., 2004; Celik et al., 2008). These mechanisms may contribute to the brain aging process by accelerating cerebral atrophy and reducing cognitive capacity (Abbott, 2002). A longitudinal study showed that elderly subjects with T2D had a greater risk of developing amnesiac mild cognitive impairment, the transitional state between normal cognitive functioning and Alzheimer's disease, as compared to elderly individuals without diabetes (Luchsinger et al., 2007; et al., 2005).

1.3 Structure and function of the blood-brain barrier

The BBB forms discrete microenvironments within the brain to support optimal functioning of a diverse array of neurotransmitters (Huber et al., 2001; Hawkins and Egleton, 2008). With over 20 m² of surface area, BBB endothelial cells serve as the physical interface between the systemic circulation and brain parenchyma (Ohtsuki and Terasaki, 2007; Pardridge, 2007; Hawkins et al., 2007; Persidsky et al., 2006; Pan and Kastin, 2004). The BBB is a semi-permeable membrane with unique characteristics that confer distinct properties that differentiate the BBB from peripheral capillaries, including a well-defined basement membrane, presence of tight junctions, absence of fenestrations, and close apposition to other brain cell types, including pericytes, astrocytes, microglia, and neurons (Huber et al., 2001). Several reviews detail the transport properties of the BBB as they pertain to both drug delivery and pathology (Toborek et al., 2003).

Once considered a static, rigid wall, the BBB is now considered a dynamic, complex structure capable of rapid modulation and responsiveness to stimuli (Winkler et al., 2001). Being a dynamic barrier allows the BBB to maintain and regulate brain homeostasis and compensate for fluctuations in the systemic circulation and increased metabolic functions within the brain; however, it also has important implications for the development and progression of central nervous system diseases, such as HIV encephalitis (Plumb et al., 2002), meningitis (Zlokovic, 2002; Ariga et al., 1998), multiple sclerosis (Saija et al., 1992), Alzheimer's and Parkinson's diseases (Latour et al., 2004), epilepsy (Brooks et al., 2005; Witt et al., 2003; Huber et al., 2002), and stroke (Crone and Christensen, 1981). Moreover, previous studies have shown that the BBB is responsive to external stimuli and systemic-based diseases (Bazzoni and Dejana, 2004; Harhaj and Antonetti, 2004). Viewing the endothelium of the BBB as part of a larger, integrated

functional unit (neurovascular unit) has opened up exciting avenues for translational, collaborative research in which the BBB is viewed as susceptible to pathology and a potential therapeutic target.

A particularly novel aspect of BBB structure is the presence of tight junctions, which create a barrier to paracellular diffusion of solutes between adjacent endothelial cells. The tight junction consists of the transmembrane proteins junctional adhesion molecule, occludin, and claudins, linked via accessory proteins including zonula occludens-1 and -2 to the actin cytoskeleton, as shown in Figure 1.1. Transmembrane proteins claudin 5 and occludin homotypically bind the adjacent endothelial cell to form the tight junction. Transmembrane proteins claudin 5 and occludin homotypically bind to form the tight junction. The BBB possesses a high electrical resistance ($1500\text{--}2000\ \Omega\cdot\text{cm}^2$), which creates both an electrical and physical barrier to maintain brain homeostasis (Huber et al., 2001).

Tight junctions are dynamic structures, in which multiple signaling pathways and factors regulate the expression, localization, and protein-protein interactions of the tight junction (Matter et al., 2005). Studies have shown that changes in total expression and subcellular localization of the tight junction proteins have been associated with alterations in paracellular permeability (Kumagai et al., 1995) and changes in localization of some tight junction proteins may play an important role in communicating the state of cell–cell contacts to the nucleus and participating in regulation of growth, differentiation, and gene expression (Zhang et al., 2001;1998).

1.4 Glycemic control, cognition, and the BBB

Many pathophysiological complications associated with diabetes, such as hyperglycemia, hypertension, and dyslipidemia have profound effects on vascular function. Pinpointing the contributions of each factor to the overall vascular dysfunction observed during diabetes is still controversial. Altered glucose transport activity at the BBB has been reported for a number of metabolic and pathophysiological conditions (Pereira et al., 2006;Schrauwen-Hinderling et al., 2007;Yorek, 2003) and two large scale, controlled clinical studies (Diabetes Control and Complications Trial and UK Prospective Diabetes Study) provide epidemiological evidence that hyperglycemia is the primary factor in the occurrence and severity of vascular complications (Gonder-Frederick et al., 1997). Research on the association between chronic hyperglycemia and increased vascular damage has focused on glucose dysmetabolism and the establishment of an imbalance between generating of reactive oxygen species and antioxidant defense enzymes and substrates, which lead to increased oxidative stress, mitochondrial dysfunction, and inflammation in the endothelial cells and surrounding tissue (Zhang et al., 2001;Zammitt et al., 2008;Anderson et al., 2006).

Hypoglycemia produces a number of adverse effects on emotion and cognition (Zhang et al., 2001;Abdelmalik et al., 2007;Frier, 2008;Velisek et al., 2008). When blood glucose levels are reduced to levels below 3 mM, such as may occur following insulin administration, subtle cognitive deficits are noted in humans (Holmes et al., 1983). Prolonged or severe hypoglycemia can lead to seizures, coma, and permanent brain damage (Cryer, 2002). Patients with T1D with episodic hypoglycemia showed signs of slowed reaction time and impaired decision making (especially involving complex tasks) (Scheepers et al., 2004). While cognitive

deficits from hypoglycemia are more prevalent in T1D, people with long standing insulin treatment for T2D have also been shown to have decreased cognition (Maran et al., 1995).

Transport of glucose from the blood across the BBB and into the cells of the brain requires a continuous, facilitative transport of glucose, which is accomplished via the expression of glucose transporter proteins (GLUT). Currently, fourteen isoforms of GLUT have been identified (McCall et al., 1986) and GLUT1 and GLUT3 are the predominate isoforms in the brain. The 55 kDa GLUT1 protein is highly expressed at the BBB and GLUT3 is primarily localized on neuronal cells within the brain. Under normal physiological conditions, GLUT1 expression at the BBB and total blood glucose levels are controlled due to tight glycemic regulation by the liver and pancreas. Brain glucose levels are maintained by an efficient homeostatic system within a narrow range, sufficient to maintain optimal neuronal function (Simpson et al., 1999; Kumagai et al., 1995). However, GLUT receptor expression changes when blood glucose levels are not regulated properly during diabetes.

There is general agreement that chronic hypoglycemia leads to increased glucose transport into the brain in animal models. Previous studies showed that chronic hypoglycemia led to increased brain glucose levels (Hou et al., 2007; Mooradian and Morin, 1991; Gjedde and Crone, 1981) associated with increased mRNA and protein expression of the 55 kDa isoform of GLUT1 on isolated cerebral microvessels (Pardridge et al., 1990). These findings suggest that upregulation of GLUT1 is an adaptive mechanism to maintain adequate glucose levels in the brain.

Effects of chronic hyperglycemia on glucose transport across the BBB have been more controversial. Several studies have demonstrated that blood-to-brain transport of glucose may be

down regulated in uncontrolled diabetes (McCall et al., 1984) with a concomitant decrease in GLUT1 expression on brain microvessels (Simpson et al., 1999) and decreased glucose metabolism (Simpson et al., 1999;Harik and LaManna, 1988). However, other studies suggest no change in GLUT1 expression on the luminal surface of cerebral microvessel (Lutz and Pardridge, 1993) and no change in glucose concentration in the brain (Badr et al., 2000). While methodological differences and degree and duration of hyperglycemic episode may play a role in the different responses, the findings that insulin treatment normalizes glucose transport and GLUT1 protein expression (Hasselbalch et al., 2001;Fanelli et al., 1998;Gutniak et al., 1990;Brooks et al., 1986) and that GLUT1 protein levels decrease in retinal microvessels after hyperglycemia (Jacob et al., 2002;Mayhan, 1997;Taarnhoj and Alm, 1991) lend a compelling argument that glucose transport across the BBB is altered to some extent. However, several studies in humans indicate no change in blood-brain glucose transport during chronic hyperglycemia and/or uncontrolled diabetes (Mayhan, 1997;McCall et al., 1984;Knudsen et al., 1986). These functional studies show the subtlety in changes observed at the BBB during diabetes, especially when compared to the microvasculature of other tissues. These studies also show the difficulties involved in mimicking the disease and translating the findings to humans; however, they also suggest that small, progressive changes in BBB function may affect neural function and, subsequently, cognition.

1.5 Diabetes and the BBB

Aside from changes in blood-to-brain glucose transport as discussed above, diabetes produces a number of changes in BBB transport function. While some transport functions, such as amino acid transport and lactate, appear to be unaltered at the BBB during diabetes (Jacob et al., 2002; Mayhan, 1997; Taarnhoj and Alm, 1991), several studies report altered transport of vital ions (K⁺, Na⁺) (Mayhan, 1997; Knudsen et al., 1986; McCall et al., 1984), and decreased choline transport into the brain. Moreover, transport of several peptides including insulin, leptin, amyloid beta and urocortin, appear to be altered in mice with streptozotocin (STZ)-induced diabetes (Kastin and Akerstrom, 2001; Kastin and Akerstrom, 2001; Banks et al., 1997; Hong et al., 2009). Of particular importance to the elderly population, who are often on several medications, diabetes has been shown to alter efflux mechanisms. A couple of recent studies show that streptozotocin-induced diabetes leads to a decrease in both mRNA and protein expression of the p-glycoprotein (multidrug resistance 1 protein; mdr1) at the BBB (Liu et al., 2006; Hawkins et al., 2007). The decreased ability to transport substrates with affinity for mdr1 may have enormous detrimental consequences, as not only would people with diabetes have lessened homeostatic control of the brain parenchyma but the brain would be at an increased risk of allowing entry of potentially neurotoxic substrates (Rechthand et al., 1987; Ennis and Betz, 1986; Knudsen et al., 1986). On the other hand, other pharmacological agents may have lessened entry into the brain due increased efflux, as noted by Hawkins et al. (2007), who found increased expression of the multidrug resistance 2 protein (MRP2) in rats with streptozotocin-induced diabetes at 7 and 14 days (Hawkins et al., 2007; Huber et al., 2006).

An area of growing interest encompasses the effect of diabetes on BBB permeability. Early reports noted that diabetes had little to no effect on blood-barrier permeability (Starr et al.,

2003). However, recent studies appear to contradict these findings in both animal models of diabetes (Kaya et al., 2004; Tomkins et al., 2001; Huber et al., 2006) and MRI evaluation of patients with diabetes (Bouchard et al., 2002). The disparity in findings may be accounted for by different measures of BBB assessment. Many studies measure BBB disruption by increased permeability of the microvasculature to albumin (Starr et al., 2003c). It can be argued that by the time albumin, a 65,000 Da protein, is measurable in the brain parenchyma, the BBB is tremendously compromised. Rather, we contend that changes in BBB function using much smaller vascular space markers, such as sucrose (342 Da) and inulin (5,000 Da), provide an intriguing opportunity to investigate the regulatory properties of the tight junction and adjacent extracellular matrix during a pathological insult and may identify future therapeutic targets. Morphologically, BBB microvasculature shows signs of diabetes-induced angiopathy, with increased vesicle formation and serum albumin staining in the Virchow-Robin space (Huber et al., 2006o). Recent studies demonstrated that small “openings” in the BBB can have a significant impact on BBB function and structure. Using magnetic resonance imaging on patients with T2D, investigators showed increased BBB permeability to gadolinium-diethylenetriamine pentaacetic acid (DTPA). These findings suggest that openings in the BBB to a small molecule (gadolinium-DTPA; 570 Da) may play a role in the progressively worsened cognitive impairment often seen in patients with diabetes (Baborie and Kuschinsky, 2006a).

When we assessed changes in BBB permeability to various sized substrates, we noted no change in albumin extravasation; however, progressive changes in permeability to smaller vascular space markers (sucrose: 342 Da and inulin: 5,000 Da) were noted (Heckmann et al., 2003; Mizushima and Seki, 2002; Huber et al., 2006). Furthermore, we reported that BBB permeability changes were regional rather than global. This finding should not be surprising as

cerebral blood flow and capillary density are not evenly distributed in white and gray matter areas of the brain (Ramakrishnan et al., 2004; Sounvoravong et al., 2004). Under basal conditions, areas with higher metabolic need (i.e. greater demand for glucose) have greater capillary density and increased cerebral blood flow. However, the area of the brain affected has a large influence on the potential for recovery. Our results suggest a differential susceptibility to diabetes-induced BBB disruption in specific brain regions. BBB disruptions in the midbrain, an area with lower capillary density and cerebral blood flow than the cortex, were observed at 28 d and were larger in size than seen in other brain areas. Clinical case reports cite an increased susceptibility to third nerve palsies in patients with diabetes due to an increased prevalence of midbrain lesions and hemorrhaging (Huber et al., 2006). Furthermore, diabetes-induced lesions have been reported to attenuate morphine analgesia due to decreased serotonergic activity in the raphe magnus nucleus (Mayhan and Heistad, 1985). Of particular interest in this investigation was the finding that other brain areas with greater cerebral flow were affected as diabetes progressed to 56 and 90 days (Huber et al., 2006). Gaining understanding of the mechanisms by which alterations in BBB permeability to normally impermeant substrates occurs has focused on changes in the regulation and/or disruption of the tight junction complex.

Traditionally, it has been proposed that BBB disruption results in increased transcellular transport rather than paracellular transport (Antonetti et al., 1998a); however, recent studies suggest that tight junctions are physiologically regulated resulting in possible increases in molecular weight-dependent paracellular flux. The association between decreased occludin expression at the tight junctions between cerebral endothelial cells and increased BBB permeability has been shown following a number of pathologies (Chehade et al., 2006). Decreased occludin expression following streptozotocin-induced diabetes has been reported in

both retinal (Hawkins et al., 2007) and cerebral (Pricci et al., 2003; Baird et al., 2002; Prasad, 2000; Hachinski et al., 1992) microvasculature. Furthermore, alterations in tight junction protein structure appear to be regulated, in part, by increased matrix metalloproteinase activity at the basement membrane (Huber et al., 2006). These findings suggest that diabetes-related changes to the CNS (i.e., increased oxidative stress, decreased vascular reactivity, and altered access to metabolic substrates) may have a cumulative effect that takes a greater period of time to manifest in altered BBB function than disruption to other vascular areas.

Another potential mechanism by which BBB function can be altered is neurovascular uncoupling caused by regional changes in cerebral blood flow. A number of factors associated with diabetes have been found to play an integral role in cerebrovascular changes, including oxidative stress, hyperglycemia, atherosclerosis, hypertension, and autonomic dysfunction (Koehler et al., 2006; Girouard and Iadecola, 2006). A recent study showed no change in global cerebral blood flow at any time point assessed following streptozotocin-induced diabetes in rats (Rosengarten et al., 2001); however, what cannot be extrapolated from these findings are possible regional breakdowns in cerebral autoregulation (e.g. neurovascular uncoupling) and the cerebral vascular response to further stressors, such as an acute hypertensive state or additional oxidative stress in the diabetic groups. The "neurovascular unit," which is composed of cerebral endothelial cells, pericytes, glia, and neurons, carefully orchestrates localized changes in cerebral blood flow to rapidly meet metabolic demands. Under basal physiological conditions, the spatial and temporal relationship between neural activity and cerebral blood perfusion, termed neurovascular coupling, utilizes cerebrovascular changes induced by activation to map regional changes in function in the human brain (Girouard and Iadecola, 2006; Parihar and Brewer, 2007; Boado, 1998; Mooradian, 1987). Moreover, cerebral blood perfusion is maintained in a

narrow range by autoregulatory mechanisms, irrespective of changes in systemic blood flow (Faraci, 2005;Egleton et al., 2003). However, in several brain pathologies, interactions between neural activity and cerebral blood vessels are disrupted, and the resulting homeostatic unbalance, known as neurovascular uncoupling, may contribute to brain dysfunction, including but not limited to BBB disruptions. In Alzheimer's disease, hypertension, and ischemic stroke, cerebrovascular function is altered, resulting in reduced cerebral blood perfusion, altered autoregulation, disruption in nutrient trafficking across the BBB, and attenuated response to increased metabolic demand (Harman, 1993). Often, these changes in cerebrovascular function precede onset of any cognitive impairment, suggesting a role for neurovascular uncoupling in the etiology and progression of cognitive dysfunction. Using these concepts, we argue that observed regional changes in BBB function may be due to a number of factors (differential neuronal viability, increased metabolic demand, and oxidative stress) in specific brain regions brought about by diabetes-associated effects, including hyperglycemia, dyslipidemia, and increased cholesterol (Mayhan et al., 2008;Abete et al., 1999;Kolosova et al., 2006;Carney et al., 1991;Reaven et al., 1999).

1.6 Accelerated aging in the diabetic brain due to elevated ROS

The free radical theory of aging (Park et al., 2007; Crivello et al., 2007) contends that aging promotes oxidative stress due to accumulation of reactive metabolic by-products and decreased activity of antioxidant enzymes. This theory has been widely substantiated by findings of decreased antioxidant enzyme activity (An-Tao et al., 2006; Kuller et al., 2007), increased reactive oxygen species (ROS) (Brands et al., 2006), and accumulation of reactive metabolic by-products (Azhar et al., 1995; Reaven et al., 1999; Carney et al., 1991; Knight et al., 1987). Similarly, increased oxidative stress on the diabetic brain results from decreased antioxidant capacity, elevated ROS, and increased pro-oxidant by-products (Celik and Erdogan, 2008; Yanardag and Tunali, 2006; Kuhad et al., 2008; Ashokkumar et al., 2006). Oxidative mechanisms may explain accelerated aging of the diabetic brain as denoted by changes in metabolism, structure and function. The implications of oxidative stress on microvascular dysfunction will be discussed in the next section.

Brain atrophy commonly occurs during the aging process (Musen et al., 2006) and has been correlated to reduced cognitive capacity. Cortical atrophy (Brands et al., 2006) and decreased grey matter (Wessels et al., 2006) have been documented in the diabetic brain. Meanwhile, these structural changes have coincided with various cognitive deficiencies (i.e. decreased attention, mental flexibility, etc.) in patients with diabetes (Cardenas et al., 2009; Moorthy et al., 2005) and the elderly (Kamboj et al., 2008; Schmatz et al., 2009). Accumulation of toxic advanced glycation end products (Takeuchi et al., 2000) and increased lipid peroxidation (Kivatinitz et al., 1997) in post-mitotic cells like neurons may lead to

apoptosis or necrosis. Neuronal death reduces cognitive capacity in the aging and diabetic populations) (Biessels et al., 2002).

Oxidative stress can alter redox sensitive transcription factors (i.e. NF κ B or HIF-1 α), affect pathway signaling by altering co-factor bioavailability (i.e. tetrahydropterin or ascorbate), or deplete energy sources (i.e. ATP or NADPH). Such changes could alter neurotransmitter receptor expression, and neurotransmitter bioavailability or synthesis. Impaired cholinergic activity has been noted in aged rats and humans (Bohnen et al., 2009; Jolitha et al., 2009). Meanwhile, acetylcholine esterase is upregulated in the brain during experimentally-induced diabetes (Kumar et al., 2008). Acetylcholine esterase inhibitors have been used for the clinical treatment of neurodegenerative diseases like Myasthenia Gravis and AD (Tripathy A, 2008) and may be beneficial for treating depressed cholinergic transmission during diabetes. Monoamine oxidase (MAO) produces hydrogen peroxide and decreases bioavailability of neurotransmitters like dopamine or serotonin. Elevated hydrogen peroxide has been documented in both the diabetic (Vincent et al., 2004) and aged brain (Archer et al., 2008). Consequently, low dopamine levels may contribute to cognitive processing deficiencies or depleted serotonin levels may play a role in increased incidences of depression observed in the aging and diabetic population (Dotson et al., 2009; Collins et al., 2009).

It is known that natural brain aging encompasses changes in structure, function and metabolism. Over time, these neurophysiological changes may reflect detrimental alterations in structural cognitive reserve and functional cognitive abilities. While diabetic encephalopathy has been termed accelerated aging of the diabetic brain, the oxidative mechanisms contributing to accelerated aging need further study. Aging mechanisms and pathological changes associated with diabetes both involve increased levels of pro-oxidants and it is no coincidence that aging

persons and patients with diabetes exhibit similar metabolic, structural and behavior changes in the brain.

1.7 Oxidative Stress and Endothelial Dysfunction

Reactive oxygen and nitrogen species (ROS and RNS) are normal by-products of metabolism that play a role in cell signaling and physiological functions. Under normal conditions, superoxide anion (O_2^-) helps regulate vascular function, cell division, inflammation, and apoptosis (Pacher et al., 2005). Superoxide is kinetically favored to combine with nitric oxide (NO^-) to create peroxynitrite ($ONOO^-$), which helps to regulate vascular smooth muscle relaxation in low concentrations. Superoxide generated from several cell sources (mitochondria, NADPH oxidases, xanthine oxidase, etc) is dismutated to hydrogen peroxide by SOD isoforms. In small amounts, hydrogen peroxide functions as vasodilatory signal to open potassium channels (Reiter, 1995). Hydrogen peroxide is converted to water by glutathione peroxidase, thiodoxin reductase, or catalase. However, when pro-oxidant levels exceed antioxidant capacity, cell damage occurs (Figure 1.2)

Excess O_2^- combines with NO^- to form damaging amounts of $ONOO^-$ that can oxidize proteins, lipids, and DNA (Reiter, 1995; Halliwell, 1992). High levels of hydrogen peroxide lead to hydroxyl anion formation, which is damaging in two ways. First, hydrogen peroxide oxidizes iron or copper which weakens the antioxidant capacity of metal-containing enzymes like SOD1, SOD2, or catalase (Stevens et al., 1993). This step forms hydroxyl anion, which initiates self-propagating lipid oxidation leading to cell membrane damage. Known as the Fenton reaction, these series of events can readily occur in the brain due to high polyunsaturated fatty acid, nonheme iron content (Stauble et al., 1994; Palumbo et al., 1992). High metabolic demand is another factor that predisposes the brain to oxidative damage. Requiring a constant supply of

oxygen and glucose, chronic hyperglycemia leads to metabolic alterations affecting brain perfusion and microvascular function.

Diabetes-induced microangiopathies involve glucose dysmetabolism which creates oxidative stress and alters homeostasis of redox cell signaling. A brief review of the pathways which may contribute to microvascular dysfunction will be included in this paragraph and is illustrated in Figure 1.3. High concentrations of glucose can be metabolized into sorbitol by aldose reductase through the polyol pathway. Increased polyol pathway activity can deplete the co-facator NADPH (Miwa et al., 2003), which is necessary for glutathione activity. In addition to decreasing antioxidant activity, sorbitol production disrupts the intracellular osmotic balance, thus creating more oxidative stress (Stevens et al., 1993). Pro-oxidants increase diacylglycerol production (DAG) (Sato et al., 2006a; Takeuchi and Yamagishi, 2009) or modulate regulatory domains of protein kinase (PKC) (Gopalakrishna and Jaken, 2000), which lead to enhanced PKC activity. PKC activation stimulates transcription factors to up-regulate genes that promote cell permeability, stimulate neovascularization and endothelial cell proliferation (Xia et al., 1996). Chronic hyperglycemia promotes glycation reactions and nonenzymatic glycation that lead to intracellular and extracellular cross-linked proteins called advanced glycation end products (AGEs) (Stitt and Curtis, 2005). AGE accumulation and receptor for advanced glycation end products (RAGE) activation has been implicated in the pathogenesis of cerebral microvascular complications and AD (Takeuchi and Yamagishi, 2009). Lastly, decreased glyceraldehydes dehydrogenase (GAPDH) leads to increased activity of the hexosamine pathway which yields UDP-N-acetylcucosamine, a post-translational modification of transcription factors (Du et al., 2003). GAPDH inhibition can result in elevated glyceralde-3-phosphate leading to the AGE precursor methoxyglycol (Beisswenger et al., 2003; Brownlee, 2005).

Chronic hyperglycemia promotes acute changes in cellular metabolism. Over time, these changes can alter homeostasis of redox sensitive cell signaling, promote accumulation of metabolic by-products (i.e. lipid peroxides, protein carbonyls, AGEs, etc.), and weaken antioxidant defenses. As a result, cerebrovascular dysfunction ensues leading to increased BBB permeability, neurovascular uncoupling, and permanent brain damage.

1.8 Antioxidant Treatment and Microangiopathies

The DCCT established that good glycemic control is the most effective method for decreasing diabetes complications in T1D patients (Greene et al., 1992; Molitch et al., 1993). Another study showed that glycemic control in children with T1D was closely associated with improved oxidant-antioxidant capacity and reduced levels of oxidative biomarkers (lipid peroxides, protein carbonyls, and 8-hydroxy-deoxyguanosine) (Dominguez et al., 1998), which may initially trigger microvascular dysfunction. Research shows that microvascular damage during diabetes is linked to both decreased antioxidant enzyme activity and elevated oxidative stress (Son, 2007; Hodgkinson et al., 2003). Therefore, it has been proposed that natural antioxidants may serve as an adjuvant therapy in the treatment or prevention of microangiopathies. Antioxidant compounds can reduce pro-oxidant damage by directly neutralizing radicals and preventing further cell damage (Sabu et al., 2002b; Keenoy et al., 1999; Ansari et al., 1998) or indirectly by enhancing antioxidant enzyme activity through essential co-factor replenishment or up-regulating gene expression) (Sadi et al., 2008; Sagara et al., 1996; Lubec et al., 1997; Piotrowski et al., 2001).

One of the most extensively studied antioxidants during diabetes is α -lipoic acid. After uptake into cells, α -lipoic acid is reduced to dihydrolipoic acid, which serves as a cofactor to regenerate antioxidants like vitamin C, vitamin E, and glutathione peroxidase (Singh and Jialal, 2008b). Studies using STZ-induced diabetic rodents in retinopathy models show that α -lipoic acid treatment maintains antioxidant enzyme capacity in the lens (Obrosova et al., 2000), prevents lipid peroxidation in the retina (Obrosova et al., 1998), maintained blood retinal-barrier integrity (Johnsen-Soriano et al., 2008) and restores retinal function (Guillonneau et al., 2003). Similar neuropathy models reveal lipoic acid improves nerve conduction deficits (Cameron and

cotter), and maintains peripheral nerve conduction and blood flow (Stevens et al., 2000; Coppey et al., 2001). Being able to cross the BBB (Piotrowski et al., 2001), α -lipoic acid decreases caspase-3 activation in neurons in rats with experimental T1D (Ametov et al., 2003; Burekovic et al., 2008). Currently, Germany has licensed α -lipoic acid as a clinical treatment for patients with diabetes. Human studies show α -lipoic acid improves antioxidant enzyme capacity and decreases oxidative stress even in patients with poor glycemic control (Borcea et al., 1999). Furthermore, α -lipoic acid improves neuropathic symptoms and nerve function in patients with T1D and T2D (Burekovic et al., 2008).

Vitamin E is a lipid-soluble antioxidant present in the plasma membranes of all cells. Diabetic rat models show that vitamin E supplementation improves fatty acid metabolism while decreasing tissue lipid peroxidation (Celik et al., 2002), and improves blood flow and nerve conduction to the heart (Rosen et al., 1995). Additionally, astrocyte activation in diabetic rats was inhibited with vitamin E treatment. Both rat and human studies show reduced low-density lipoprotein oxidation (Fuller et al., 1996; Li et al., 1996). Human studies reveal that vitamin E and vitamin C supplements reduced oxidative stress in the eye and improves vascular endothelial function in T1D, but not T2D patients (Peponis et al., 2002). Furthermore, topical vitamin E improves skin microcirculation in T2D patients (Ruffini et al., 2003).

Polyphenols are dietary antioxidants typically found in fruits, vegetables, and teas. Green tea, possessing different polyphenols, inhibits lipid peroxidation and scavenges hydroxyl and superoxide radicals (Sabu et al., 2002). The phenolic antioxidant mechanism is based on the donation of a hydrogen and formation of a phenoxyl radical which stabilizes itself by releasing a further hydrogen or by reaction with another radical (Do et al., 2003). When green tea is given to diabetic rats, SOD and GSH levels increase and retinopathy is improved by reduced acellular

capillaries and pericyte ghosts (Mustata et al., 2005). Recent studies show that phenolic compounds play an important role in protecting the brain from oxidative damage due to their abilities to cross the BBB (Andres-Lacueva et al., 2005;Mandel et al., 2006).

1.9 Sesamol

Sesamol (Figure 1.4a), a natural antioxidant found in sesame seed oil, is a synthetic precursor to the selective serotonin reuptake inhibitor paroxetine (Figure 1.4b). Interestingly, paroxetine has been identified as a beneficial therapy in treating diabetes-associated neuropathy (Sindrup et al., 1990; Sindrup et al., 1991). Furthermore, sesamol improved diabetes-associated cognitive decline (Kuhad and Chopra, 2008) and improved BBB structure and function (VanGilder et al., 2009) in STZ-diabetic rats. If diabetes plays a role in vascular associated dementia, then sesamol and sesamol-derivatives may be advantageous in preventing cerebral microangiopathy.

Few studies have examined the pharmacokinetic properties of sesamol; however, the data available is comparable to the pharmacodynamic parameters of paroxetine. Both compounds are transported by plasma proteins; sesamol is 55% bound and paroxetine is 95% bound (Bryson and Bischoff, 1970b; Haddock et al., 1989). However, with sesamol having solubility in aqueous and lipophilic phases, 25% of sesamol can be found in aqueous blood components (Bryson and Bischoff, 1970). Both compounds undergo first pass hepatic metabolism which yields glucuronide and sulfate metabolites (Hou et al., 2008; Jan et al., 2008; Kaye et al., 1989). Paroxetine is 85% metabolized and its metabolites lack bioactivity (Kaye et al., 1989). The approximate bioavailability of sesamol lies between 36-46% with systemic sesamol levels being ~5% of the metabolite content (Hou et al., 2008; Jan et al., 2009). Jan (2008) proposed that the glucuronide and sulfate metabolites of sesamol lack bioactivity; however, Hou (2008) suggested that further studies are needed. Elimination of sesamol and its metabolites occurs within 24 h (Jan et al., 2008); conversely, the terminal half life of paroxetine is 24 h (Haddock et al., 1989).

Data suggest that sesamol, unlike paroxetine, does not accumulate in the body, indicating that several daily doses are needed if sesamol were used for clinical treatment of oxidative stress. Sesamol toxicity studies have not been published; however, multiple daily dosings of 50 mg/kg (s.c.) or one dose of 100 mg/kg (s.c.) have safely been given to rats or mice, respectively (Hsu et al., 2007; Hou et al., 2008).

Sesamol, but not its metabolites, has been identified in brain tissue (Jan et al., 2008). This could be attributed to its low molecular weight and lipophilic nature, which makes sesamol a primary candidate for crossing the BBB. Despite having chemical properties favoring BBB passage, the amount of sesamol entering the brain may be limited due to PGP activity as paroxetine has been noted as a PGP substrate (Uhr et al., 2003). In the brain, sesamol likely exerts its antioxidant effects through two mechanisms. First, sesamol is able to scavenge superoxide anion (Hsu et al., 2006; Aboul-Enein et al., 2007; Joshi et al., 2005), a property attributed to phenolic compounds. The benzodioxyl moiety gives sesamol the unique ability to neutralize hydroxyl anion (Hsu et al., 2008; Joshi et al., 2005; Hiramoto et al., 1996) and peroxy radical (Parihar and Pandit, 2003; Uchida et al., 1996; Gupta et al., 2009). Second, the actions of the benzodioxyl group may include gene regulating abilities, as shown by sesamol and other benzodioxyl-containing compounds (Tenorio-López FA, 2007; Schneider K Keller S, 2008; Jurd et al., 1987). Sesamol attenuates the production of nitric oxide (Chen et al., 2005) and hydrogen peroxide and reduces MAO activity in cultured astrocytes (Mazzio et al., 1998). Alterations in MAO activity correlate to oxidative stress and neurodegenerative disease development seen in aging, AD and stroke. Likewise, defects in fibrinolysis play a role in cardiovascular disease and increased cerebrovascular disease risk. Sesamol may enhance fibrinolytic capacity through modulating plasminogen activator gene expression (Chen et al., 2005). *In vivo* experiments

confirm the antioxidant abilities of sesamol and many sesamol-related lignans can also alter gene regulation. However, gene regulation studies on sesamol have only been done *in vitro*; therefore, further research is warranted.

1.10 Contribution of Matrix Metalloproteinases to BBB Dysfunction

Matrix metalloproteinases (MMPs) are a family of Ca^{2+} - or Zn^{2+} - containing endopeptidases that are typically released as a pro-zymogen and catalytically converted to the active zymogen. MMPs have three similar structural domains: an aminoterminal propeptide, a catalytic metal-catalytic domain and a hemopexin-like domain at the carboxy-terminal end. A subtype of MMPs are known as the gelatinases. Gelatinase A (MMP-2) and gelatinase B (MMP-9) degrade basement membrane (e.g. laminin and heparan sulfate proteoglycans) and extracellular matrix (e.g. collagen IV and fibronectin) proteins, which make their role essential for controlling cell growth, survival and differentiation, extracellular matrix (ECM) remodeling, processing of growth factors expressed on the ECM (Agrawal et al., 2008), and modifying brain synapses (Ethell and Ethell, 2007).

MMP tissue expression is low under physiological conditions. However, during a pathological state like diabetes, elevated ROS can initiate increased MMP protein levels and activity (Kim et al., 2007; Shin et al., 2008; Galli et al., 2005; Haorah et al., 2008). Increased ROS can influence redox sensitive translation factors to upregulate gelatinase transcription and/or expression (Kim et al., 2003; Machado et al., 2006; Gu et al., 2005; Hawkins et al., 2007; Thrailkill et al., 2007; Signorelli et al., 2005). Likewise, nitration or oxidation of the inhibitory cysteine residue prematurely activates the enzyme (Gasche et al., 1999; Giebel et al., 2005). ROS-induced gelatinase activity degrades basement membrane protein leading to decreased stability and integrity of the endothelium *in vivo* and *in vitro* (Arrick et al., 2008).

While dysregulated MMP 2 and MMP-9 activity is closely linked to diabetes-induced microangiopathies seen in the nerve, retina and kidney (Chattopadhyay et al., 2007; Kowluru and

Kanwar, 2009;Thraikill et al., 2009), less is known about the contribution of gelatinase activity to cerebral microvasculature. Clinical and experimental diabetes studies have shown higher circulating MMP-2 and MMP-9 levels in blood serum (Hawkins et al., 2007;Thraikill et al., 2007;Signorelli et al., 2005) Likewise, gelatinase activity may contribute to decreased tight junction protein expression and loss off BBB integrity as the tight junction proteins (i.e. occludin and ZO-1) have been shown to be MMP substrates (Wachtel et al., 1999;Harkness et al., 2000). *In vitro* studies indicate that BBB integrity directly correlates to oxidative stress and gelatinase activity (Haorah et al., 2007). Likewise, *in vivo* studies show that SOD-2 knockout mice (Maier et al., 2006), showing lower antioxidant capacity, exhibit greater BBB dysfunction due to elevated MMP activity. Conversely, SOD-1 overexpression reduces MMP-9 activation (Morita-Fujimura et al., 2000).

Evidence suggests that ROS activate gelatinase proteolytic activity, which may contribute to progressive cerebromicrovascular damage. Other studies suggest that gelatinase activity might be modulated with natural antioxidant administration (Swarnakar and Paul, 2009). Given this etiology, antioxidant administration or MMP inhibitors (Yaras et al., 2008) might be beneficial in reducing microvascular dysfunction.

1.11 Uncoupling of Endothelial Nitric Oxide Synthase

Physiological conditions result from a balanced release of endothelial-derived relaxing and contracting factors. Nitric oxide produced from endothelial nitric oxide (eNOS) is responsible for vessel dilation (Laughlin et al., 2003) and maintaining of vascular function (by preventing adhesion molecule expression, MMP activation and dysregulated neovascularization) (Pacher et al., 2005). eNOS plays a critical role in maintaining functional hyperemia for the brain (e.g. neurovascular coupling). When cortical and subcortical networks are recruited for performing specialized tasks, nitric oxide production dilates local vessels to increase blood flow and meet neuronal energy demands (Rosengarten et al., 2001; Haydon and Carmignoto, 2006). If the demand for glucose and oxygen are not met, mitochondrial respiration fails to produce sufficient amounts of ATP to maintain neuronal metabolic demand. A phenomenon known as neurovascular uncoupling occurs (Dahle et al., 2009; Rosengarten et al., 2001; Vicenzini et al., 2007). This section will focus on how the pathology of diabetes leads to uncoupled eNOS which may lead to neurovascular uncoupling.

Oxidative stress reduces nitric oxide bioavailability through peroxynitrite formation and promoting eNOS uncoupling. Uncoupled eNOS occurs due to decreased levels of substrate (L-arginine) (Settergren et al., 2009) or oxidized cofactors (tetrahydropterin or zinc) by peroxynitrite (Bendall et al., 2005; Zou et al., 2002). When eNOS is uncoupled, vasodilatory abilities of vessels are impaired because the nitric oxide producing dimeric form is lost and the superoxide/hydrogen peroxide generating monomeric form is favored (Alp and Channon, 2004). Decreased nitric oxide results in poor vessel dilation in T1D rats (Arrick et al., 2008) and human

T2D patients (Didion et al., 2005). Enhanced vessel constriction translates into progression of vascular disease and end-organ damage (Hadi and Suwaidi, 2007).

Poor perfusion of the brain can be particularly damaging because the brain lacks a metabolic reserve. Reduced cerebrovascular reactivity (Kozera et al., 2009) and reduced cerebral blood flow develops into vascular dementia (Vicenzini et al., 2007; Osawa et al., 2004; Bateman et al., 2006) associated with endothelial dysfunction (Beal, 1995). Uncoupled eNOS limits vascular reactivity of larger vessels leading to insufficient capillary perfusion and blood supply for neuron function, thereby promoting neurovascular uncoupling. Neurovascular uncoupling may contribute to progressive BBB damage during diabetes and certain regions of the brain appear to be more vulnerable (Huber et al., 2006). Cortical regions have a high capillary density, thus indicating a greater metabolic demand. In the cortex, BBB permeability precedes neuron loss and functional disabilities (Starr et al., 2009; DiNapoli et al., 2008; Rite et al., 2007). Due to low capillary density, subcortical regions with endothelial dysfunction show signs of ischemia (Gouw et al., 2008; Sonnen et al., 2009). Subcortical ischemic lesions are associated with an increased risk for lacunar infarcts (Karapanayiotides et al., 2004), decreased serotonergic activity (Sounvoravong et al., 2004) and global capillary loss (Brown et al., 2007). Such changes can alter neurotransmitter metabolism or bioactivity and promote neuron death.

1.12 Conclusion

Diabetes is a chronic disease requiring life-long treatment; therefore, long-term health concerns and preventative measures must be addressed. Hyperglycemia-induced ROS have been repeatedly shown to be a primary cause for microangiopathy. With poor blood glucose control and microvascular dysfunction playing a significant role in debilitating diabetes-associated complications, understanding cerebral microvasculature dysfunction will provide mechanistic insight into cognitive deficiencies. Currently, most of the research has used insulinopenic animal models, which most closely resemble type 1 diabetes; however, the vast majority of people have (insulin-resistant) type 2 diabetes. Thus, it would seem a necessary next step is to assess changes in BBB function in animal models displaying insulin resistance. Type 2 diabetes most often occurs in conjunction with other risk factors (i.e. hypertension, obesity, age) that are also correlated to elevated oxidative stress.

The unique phenotype of the BBB regulates which substances can access the neuronal microenvironment to maintain homeostasis so that neurons can properly function. Oxidative stress has been correlated to cerebromicrovascular dysfunction and neurodegenerative disease progression. While antioxidant therapy has shown beneficial effects in treating diabetes-induced microangiopathies and improving memory in neurodegenerative disease models, a mechanism between oxidative stress, microangiopathy, and cognitive dysfunction has not been strongly established. Understanding how progressive perturbations in the BBB occur may broaden the understanding of neurodegenerative disorders (stroke, AD, and multiple sclerosis) as these disease states have shown BBB permeability before significant neuron loss and permanent cognitive deficits ensue.

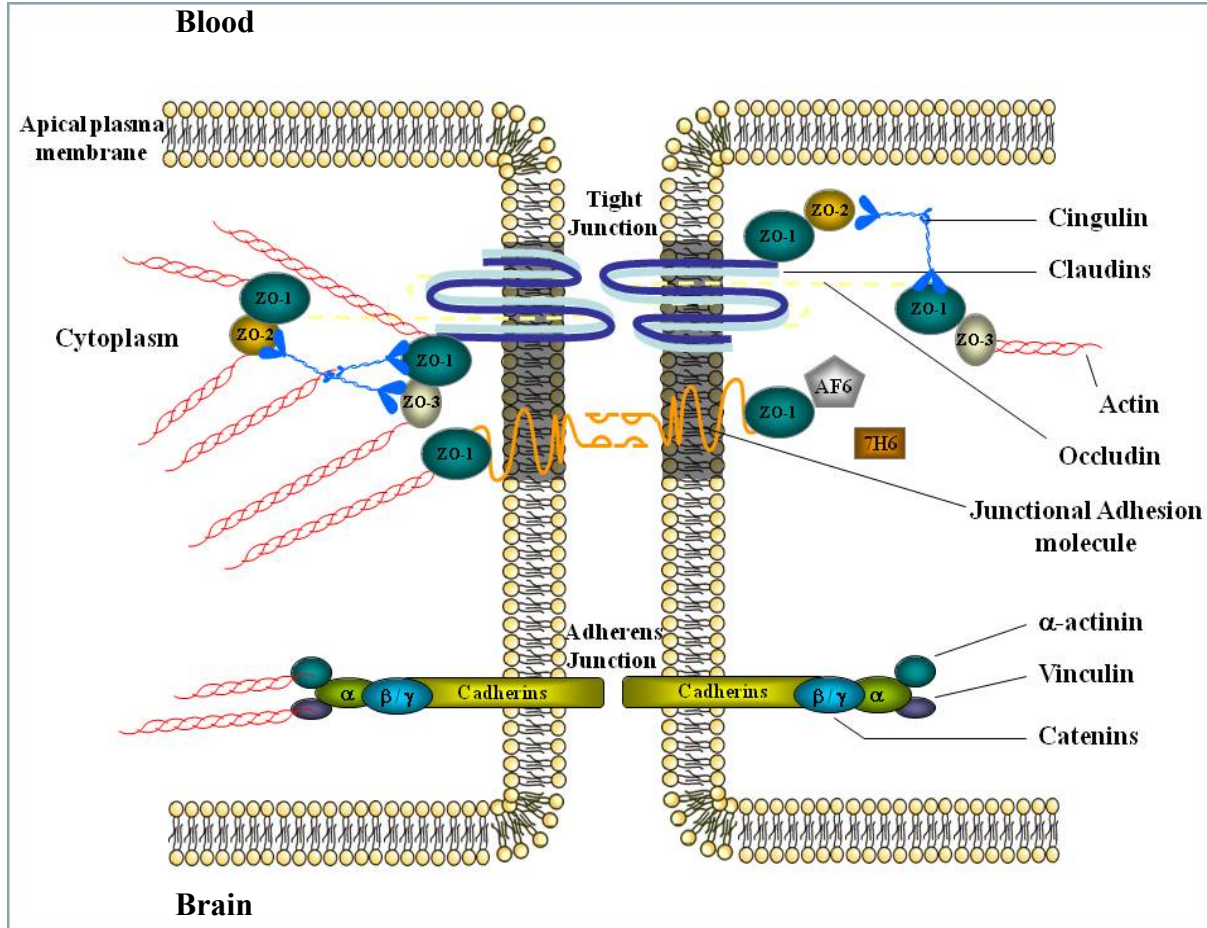


Figure 1.1 Schematic representation of intercellular junction between two endothelial cells. Cerebral endothelial cells possess cadherins, junction adhesion molecule, and tight junctions. However, the tight junction provides the cerebral endothelium with its unique phenotype. Zona occludens serve as the cytoskeletal scaffolding for the transmembrane proteins claudin-5 and occludin, which forms the cell-to-cell junction. Claudin 5 is essential for tight junction formation. Occludin exists as a cytosolic or plasma membrane protein and location within the cell reflect changes in paracellular permeability (Huber, 2001)

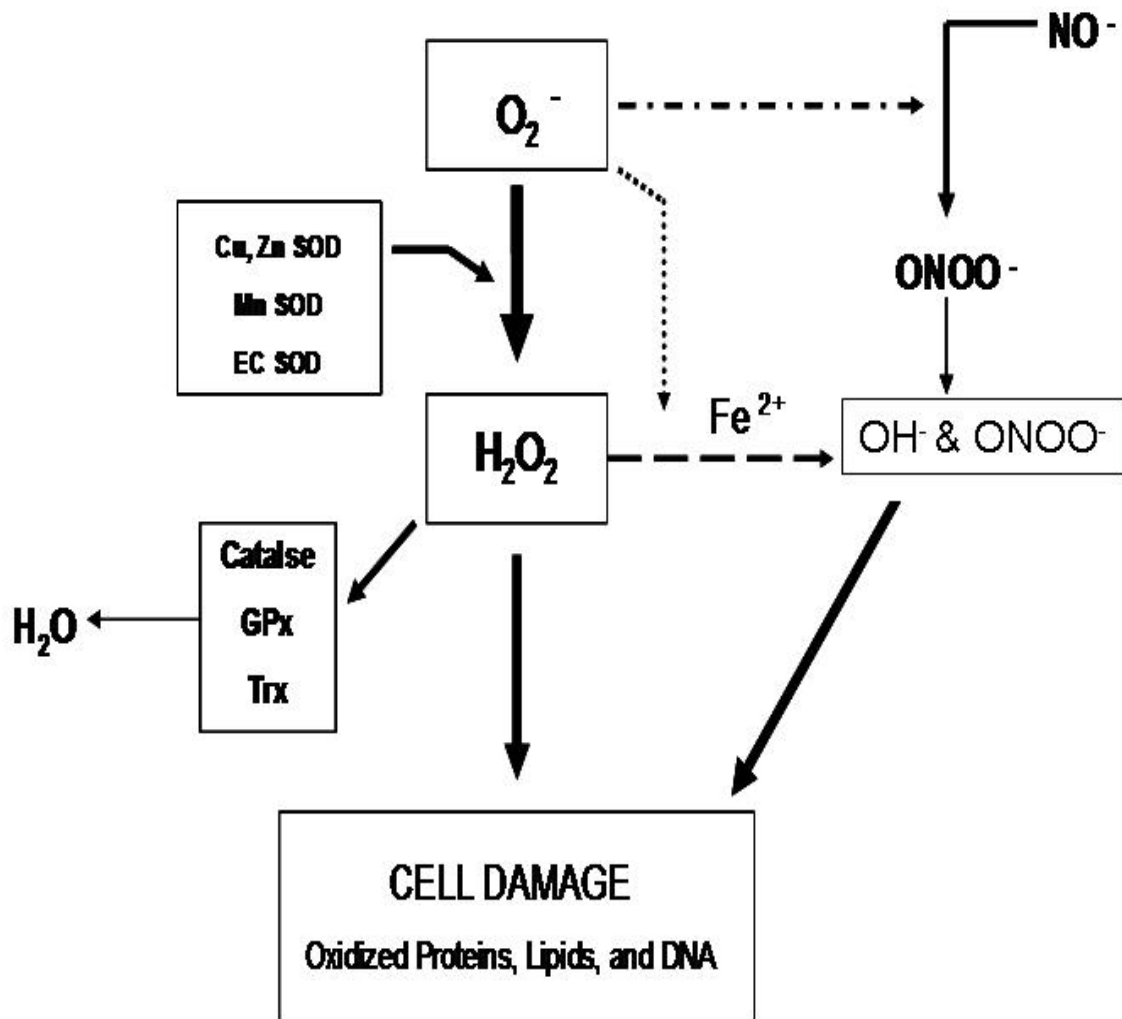


Figure 1.2 Diagram illustrating how ROS are reduced by antioxidant activity or lead to cell damage. Superoxide anion gets transformed to hydrogen peroxide by SOD isoforms. Hydrogen peroxide is reduced to water and molecular oxygen by catalase (CAT), glutathione peroxidase (GPx), or thiodoxin reductase (Trx). Excess superoxide reacts with nitric oxide to form peroxynitrite. Excess hydrogen peroxide can form hydroxyl anion. Peroxynitrite and hydroxyl anion lead to damaged protein, lipids and DNA.

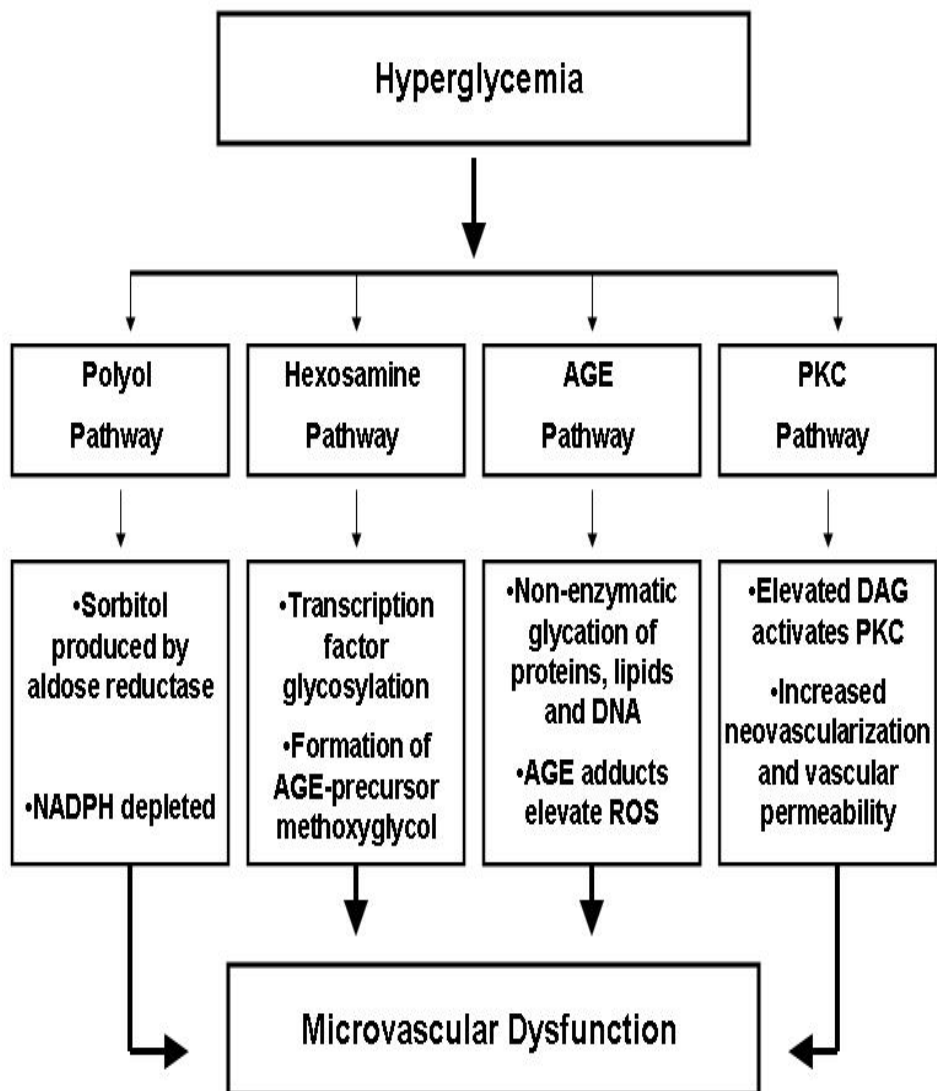


Figure 1.3 Diagram showing how hyperglycemia affects metabolic pathways and leads to microvascular dysfunction. The polyol pathway, hexosamine pathway, AGE formation, and PKC signaling are implicated as causes for diabetes induced microangiopathies. Biochemical changes leading to microdysfunction are briefly summarized.

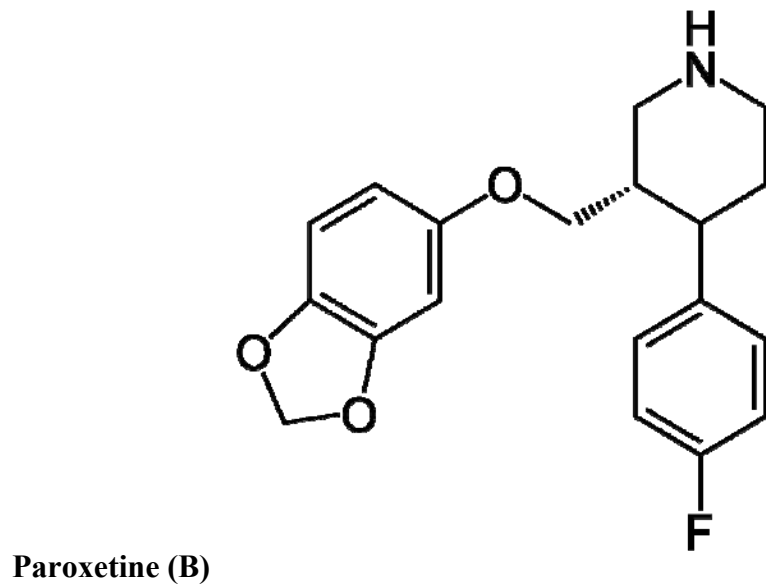
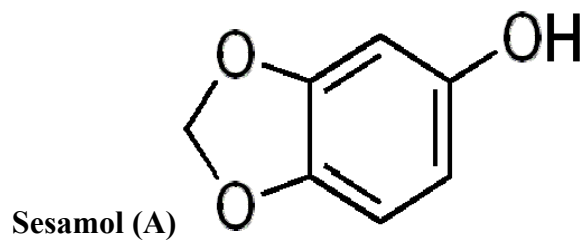


Figure 1.4 Chemical structures of sesamol (A) and paroxetine (B). Sesamol is a precursor to paroxetine and shares similar pharmacokinetic properties. The selective serotonin reuptake inhibitor paroxetine may have therapeutic benefits for treating diabetes-associated neuropathies.

CHAPTER TWO

**Streptozotocin-induced diabetes progressively increases blood-brain
barrier permeability in specific brain regions in rats**

This chapter is identical to a manuscript published in the American Journal of Physiology-Heart
and Circulation Physiology in December 2006.

2.1 ABSTRACT

This study investigated the effects of streptozotocin-induced diabetes on the functional integrity of the blood-brain barrier in the rat at 7, 28, 56, and 90 d using vascular space markers ranging in size from 342 to 65,000 Da. We also examined the effect of insulin treatment of diabetes on the formation and progression of cerebral microvascular damage and determined whether observed functional changes occurred globally throughout the brain or within specific brain regions.

Results demonstrate that streptozotocin-induced diabetes produced a progressive increase in blood-brain barrier permeability to small molecules from 28 to 90 d and these changes in blood-brain barrier permeability were region specific with the midbrain most susceptible to diabetes-induced microvascular damage. In addition, results showed that insulin treatment of diabetes attenuated BBB disruption, especially during the first few weeks; however, as diabetes progressed, it was evident that microvascular damage occurred even when hyperglycemia was controlled. Overall, results of this study suggest that diabetes-induced perturbations to cerebral microvessels may disrupt homeostasis and contribute to long-term cognitive and functional deficits of the central nervous system.

2.2 INTRODUCTION

Diabetes mellitus is a chronic progressive disease that often results in vascular complications, including the development of microangiopathy, which is characterized by basement membrane thickening (Hill and Williams, 2004d; Roy and Sato, 2000), cytoskeletal rearrangement (Yu et al., 2005) and increased paracellular leakage (Idris et al., 2004). Extensive research has been conducted on endothelial cell dysfunction in a number of tissues, including kidney, peripheral nerve, retina, heart, and skeletal muscle (Basile et al., 2004; Cukiernik et al., 2004; Gustafsson and Kraus, 2001; Hill and Williams, 2004; Pereira et al., 2006; Wada and Yagihashi, 2005). From these studies, important factors, including prolonged hyperglycemia, hypertension, increased oxidant stress, dyslipidemia, and insulin resistance, (Baird et al., 2002; Lee et al., 2002; Osicka et al., 2003; Pricci et al., 2003; Salmi et al., 2002; Thomas et al., 2004) have been shown to play a role in diabetes-induced endothelial cell dysfunction. While overwhelming evidence shows that diabetes is a disease of the vascular system, few studies have investigated the effects of diabetes on the vasculature of the central nervous system. However, recent clinical evidence suggests diabetes leads to increased incidences of vascular dementia, ventricular hypertrophy, lacunar infarcts, and hemorrhage (Appelros et al., 2005; Colwell et al., 1983; Karapanayiotides et al., 2004; Rosenkranz et al., 2003) and may be a predisposing factor for Alzheimer's disease (Ristow, 2004). Thus, with the growing prevalence of diabetes in our society, understanding how diabetes affects the vascular system of the brain is an understudied yet important area of inquiry.

The blood-brain barrier (BBB) is situated at the level of the endothelial cell and serves to partition the systemic circulation from the brain parenchyma. The BBB forms discrete microenvironments within the brain to support optimal functioning of a diverse array of

neurotransmitters (Abbott, 2002b). The BBB is characterized by a well-defined basement membrane, presence of tight junctions, absence of fenestrations, and close apposition to other brain cell types, including astrocytes, pericytes, microglia, and neurons. These unique characteristics confer distinct properties that differentiate the BBB from peripheral capillaries (Huber et al., 2001). For example, tight junctions between BBB endothelial cells lead to a high transendothelial electrical resistance of 1500-2000 $\Omega \cdot \text{cm}^2$ as compared to 3-33 $\Omega \cdot \text{cm}^2$ in other vascular tissues. The net result of this high electrical resistance is low paracellular diffusion and limited formation of transcapillary endocytosis; thus, enabling a highly regulated and stable microenvironment within the brain. Being a dynamic barrier allows the BBB to maintain and regulate brain homeostasis and compensate for fluctuations in the systemic circulation and increased metabolic functions within the brain; however, a number of CNS-associated diseases, including HIV encephalitis (Toborek et al., 2003), meningitis (Winkler et al., 2001), multiple sclerosis (Plumb et al., 2002), Alzheimer's and Parkinson's diseases (Ariga et al., 1998; Ziylan et al., 1984), epilepsy (Saija et al., 1992), and stroke (Latour et al., 2004) have been shown to disrupt BBB structural integrity leading to functional breakdown.

Many studies measure BBB disruption by increased permeability of the microvasculature to albumin (Kaya et al., 2004; Tomkins et al., 2001). We argue that by the time albumin, a 65,000 Da protein, is measurable in the brain parenchyma, the BBB is already compromised. Rather, we argue that assessing changes in BBB function using much smaller vascular space markers, such as sucrose (342 Da) and inulin (5,000 Da) provide an intriguing opportunity to investigate the regulatory properties of the tight junction and adjacent extracellular matrix during a pathological insult and may identify future therapeutic targets. Morphologically, BBB microvasculature has shown signs of diabetes-induced angiopathy, with increased vesicle

formation and serum albumin staining in the Virchow-Robyn space (Bouchard et al., 2002). Recent studies demonstrated that small openings in the BBB indicated changes in BBB function and structure, which may have a significant impact on neuronal function. Using magnetic resonance imaging on patients with type II diabetes, investigators showed increased BBB permeability to gadolinium DTPA and concluded that though the openings in the BBB were to a small molecule (gadolinium DTPA; 570 Da), clinical significance was substantial as these effects may play a role in the increased the progressive cognitive impairment often seen in patients with diabetes (Starr et al., 2003). Additionally, a recent study showed that streptozotocin (STZ)-induced diabetes in rats altered the molecular structure of BBB tight junctions by decreasing the expression of occludin, with no change in the accessory protein zonula occludens 1 (ZO-1) (Chehade et al., 2002).

Due to the progressive nature of diabetes and the unique phenotype of the BBB, the effects of diabetes on the cerebrovasculature are different from other microvascular beds and barrier systems, such as seen at the retina and peripheral nerves. Adverse effects at the BBB may be more insidious as vascular dysregulation is less perceptible at first and by the time clinical signs are noticeable, irreversible neurological damage may have occurred. We hypothesize that diabetes has a long term, progressive effect on BBB endothelial cells resulting, at first, in small, transient breaches that over time grow larger and more pronounced.

2.3 MATERIALS AND METHODS

Chemicals and Radioisotopes: STZ, regular insulin, Evan's blue, and reagent grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO). [¹⁴C]sucrose (specific activity: 485 mCi/mmol, >99.5% purity) and [³H]inulin (specific activity: 355 mCi/g, >99% purity) were purchased from MP Biomedical (Costa Mesa, CA). [³H] Butanol (specific activity: 20 Ci/mmol; >99% purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Animals: Male Sprague-Dawley rats (Harlan Sprague-Dawley; Indianapolis, IN) weighing 250-274 g were housed under 12 h light/dark conditions and received food and water ad libitum. Animals were acclimatized to the environment for 7 d before induction of diabetes. All protocols involving animals were approved by the West Virginia University Animal Care and Use Committee and abide by NIH guidelines.

Diabetic induction procedures: STZ was dissolved in sodium citrate (50 mM; pH 4.5) buffered 0.9% saline and regular insulin was dissolved in 0.9% saline. Rats were divided into three treatment groups. Group I received a single injection of sodium citrate buffered 0.9% saline and served as the control. Group II received a single injection (i.p.) of STZ (60 mg/kg; 100 μ l). Group III received a single injection of STZ (60 mg/kg; 100 μ l) and then received regular insulin (4 U/kg; s.c.) twice daily upon determination of hyperglycemia. Glucose water (10%) was put into cages of rats given STZ for 12 h to protect against STZ-induced hypoglycemia. Animals were classified as diabetic if blood glucose level measured >350 mg/dl and only animals with a blood glucose level > 350 mg/dl were allowed to continue in Groups II and III.

Experimental procedures: Animal studies were conducted at 7, 28, 56, and 90 d. Blood glucose and weight were measured prior to studies. Animals from each group were assessed for BBB permeability to Evan's blue albumin (65,000 Da), [³H]inulin (5,000 Da) and [¹⁴C]sucrose (342 Da). To determine localization of changes in BBB permeability, rat brains were dissected on ice (in the following order): hypothalamus, cerebellum, midbrain, cerebral cortex, hippocampus, basal ganglia, and thalamus.

Evan's blue extravasation: For quantification of albumin extravasation, rats were anesthetized with pentobarbital sodium (60 mg/kg; i.p.) and 2% Evans blue (4 ml/kg; 1 ml) was infused via the femoral artery and allowed to circulate for 1 h. Rats were perfused with cold phosphate buffered saline with heparin (2 U/ml; pH 7.4) for 15 min via the left ventricle. After perfusion, rats were sacrificed by decapitation and brain extracted. Excised brain was weighed, dissected, and homogenized in 500 µl of 50% trichloroacetic acid. Tissue was incubated for 24 h at 37°C. At 24 h, samples were centrifuged at 13,000 x g for 10 min and supernatants diluted 4-fold with absolute ethanol. Fluorescence intensity was measured using a spectrofluorometer at 620 nm excitation, 680 nm emission (RF 5301 PC; Shimadzu; Columbia, MD). Calculations were based on external standard readings and extravasated dye expressed as ng Evan's blue / mg brain tissue.

In situ brain perfusion: *In situ* brain perfusion studies were carried out based on method of Preston *et al.* (Preston *et al.*, 1995). Briefly, rats were anesthetized with rat cocktail (flunixin: 2.5 mg/kg, ketamine: 90 mg/kg, xylazine: 5 mg/kg; i.m.), heparinized (10,000 U/kg; i.p.), and body temperature maintained at 37°C. Common carotid arteries were exposed and right common carotid cannulated and perfused with an erythrocyte-free perfusion media consisting of a

modified Krebs-Henseleit Ringer's (117 mM NaCl; 4.7 mM KCl; 0.8 mM MgSO₄; 24.8 mM NaHCO₃; 1.2 mM KH₂PO₄; 2.5 mM CaCl₂; 10 mM D-glucose; dextran (70,000 Da) 29 g/L; bovine serum albumin 1 g/L), which was aerated with 95% O₂/5% CO₂ and warmed to 37°C. With start of the perfusion, the right jugular vein was sectioned to allow for drainage. Once the desired perfusion pressure (85-95 mmHg) and flow rate (3.1 ml/min) were achieved for right common carotid artery, the contralateral carotid artery was cannulated and perfused in a similar manner. Once both arteries were cannulated, radiolabeled compound was infused via a slow-drive syringe pump (flow rate: 0.5 ml/min; Model 22; Harvard Apparatus) into the inflowing mammalian Ringer's solution (total flow rate: 3.6 ml/min/hemisphere). After 20 min, brain was flushed for 20 s with unlabeled Ringer's solution and the animal decapitated. The brain was removed and choroid plexuses and meninges excised. The brain was dissected into brain regions as described above and homogenized. Perfusion fluid was collected from carotid cannula by briefly resuming perfusion of radiolabeled compound following termination. Brain tissue samples (~500 mg wet weight) and 100 µl of perfusate samples were prepared for radioactive counting by addition of 1 ml of tissue solubilizer (TS-2; Research Products Inc.; Mount Prospect, IL). Then 30 µl of glacial acetic acid (to quench chemiluminescence) and 4 ml of scintillation cocktail (Budget Solve; Research Products Inc.) were added and samples analyzed by liquid scintillation counting on a Beckman LS5801 (Beckman Coulter; Fullerton, CA). Amount of [³H] and [¹⁴C] radioactivity in the brain (C_{tissue}; dpm/g) was expressed as a percentage of that in the artificial perfusate (C_{perfusate}; dpm/ml) and termed R_{tissue}% (µl/g) as follows: **R_{tissue} % = (C_{tissue} / C_{perfusate}) X 100%.**

Capillary depletion studies: Capillary depletion method was based on method of Triguero *et al.* (Triguero et al., 1990). After *in situ* perfusion, brain was removed, choroid plexuses and meninges excised, dissected as described above, and homogenized in 1.5 ml of capillary depletion buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; 141 mM NaCl; 4 mM KCl; 2.8 mM CaCl₂; 1 mM MgSO₄; 1 mM NaH₂PO₄; 10mM D-glucose; pH 7.4) and kept on ice. Two ml of ice-cold dextran (60,000 Da) solution were added to homogenate. Two aliquots of homogenate were taken and centrifuged at 5,400 x g for 15 min. Capillary depleted supernatant was separated from vascular pellet. Homogenate, supernatant, and pellet were counted for radioactivity on scintillation counter. All homogenation procedures were carried out within a 2 min time span.

Measurement of cerebral blood flow: The perfusion method of Preston *et al.*, (Preston et al., 1995) was adapted to determine both CBF and rate of cerebral perfusion *in situ* to determine the [³H]butanol uptake using derived equations of Gjedde *et al.*, (Gjedde and Crone, 1981). *In situ* brain perfusion was carried out as stated above with a Ringer's solution containing 4 ml/L unlabeled ethanol. With the use of a slow-drive syringe pump (0.5 ml/min per hemisphere), [³H] butanol was added during last 10 s of a 20 min perfusion. A partition coefficient (λ_{br}) was determined using a separate group of animals (n=3) for each treatment and time that was perfused with a constant [³H] butanol concentration in arterial inflow for 20 min followed by brain sampling and analysis. After perfusion, brains were weighed and sectioned. Brain and Ringer's solution samples were taken for liquid scintillation counting. A small portion of frontal lobes (~50 mg) was removed and weighed separately to determine the brain tissue dry weight by drying in an oven at 95°C to constant weight. Unlabeled ethanol was added to saturate endogenous alcohol dehydrogenase for both measurements.

Calculation of CBF: Measurement of cerebral blood flow was quantified by using the derived equation from Gjedde et al., (Gjedde and Crone, 1981a): $F_{bl} = -R_{br} \ln[(1 - C_{br(t)} / R_{br} \times C_a) / t]$, where F_{bl} is rate of blood flow (ml/min per unit mass (g)) and C_a is the constant [^3H] butanol concentration in arterial inflow at *time t* between introduction of [^3H] butanol and decapitation. C_{br} is activity in unit weight of brain at *time t*. λ_{br} is the distribution ratio of [^3H] butanol between brain and perfusion medium at steady state. The value of λ_{br} was calculated as ratio of ^3H radioactivity in brain versus ^3H radioactivity in arterial inflow. Extraction of the tracer from blood is assumed to be complete during a single capillary pass.

Statistical Analysis: Statistical significance ($\alpha = 0.05$) for differences in BBB permeability to vascular space markers, capillary depletion, cerebral blood flow, and interaction between treatment groups and day of whole brain and brain regions were determined by two-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post hoc analyses.

2.4 RESULTS

Determination of Evan's blue extravasation: BBB permeability to albumin (65,000 Da) was measured in whole brain of age-matched vehicle treated (Group I), diabetic (Group II), and insulin-treated diabetic (Group III) rats at 7, 28, 56, and 90 d by quantification of Evan's blue. Evan's blue binds with affinity to albumin and is a commonly used tool to quantify albumin extravasation in tissue and predict edema formation. Results showed no significant ($p>0.05$) difference in total Evan's blue extravasation in whole brain between any treatment group at any time point (Figure 1A). No significant ($p>0.05$) interaction between treatment and day was observed.

Determination of regional differences in Evan's blue extravasation: Regional differences in BBB permeability to albumin (65,000 Da) were measured in rats from Groups I, II, and III at 7, 28, 56, and 90 d by quantification of Evan's blue (Table 1). Results showed that extravasation of Evan's blue was not significantly ($p>0.05$) different between Groups I, II, and III at 7, 28, and 56 d. At 90 d, Groups II and III exhibited a significant ($p<0.05$) increase in Evan's blue extravasation in the midbrain (1.0 ± 0.2 and 0.9 ± 0.1 , respectively) as compared to Group I (0.3 ± 0.1). Moreover, Group II had a significant ($p<0.05$) increase in Evan's blue extravasation in the basal ganglia (1.5 ± 0.2) as compared to Group I (1.0 ± 0.1) at 90 d. No significant ($p>0.05$) interaction between treatment and day within a region was observed.

In situ brain perfusion using [^3H]inulin: BBB permeability was assessed in whole brain of rats from Groups I, II, and III at 7, 28, 56, and 90 d after STZ-induction by *in situ* brain perfusion with an impermeant marker ([^3H]inulin; 5,000 Da) over 20 min. Results showed no significant

difference ($p > 0.05$) in sucrose associated with total brain between the treatment groups (II and III) as compared to Group I at any time point (Figure 1B). No significant ($p > 0.05$) interaction between treatment and day was observed.

Determination of regional differences in [^3H]inulin association with brain: Using *in situ* brain perfusion with [^3H]inulin, we assessed rats in Groups I, II, and III for changes in permeability of inulin into different brain regions at 7, 28, 56, and 90 d following STZ-induced diabetes (Table 2). Results showed no significant ($p > 0.05$) change in overall BBB permeability to [^3H]inulin; however, a few brain regions of Groups II and III exhibited a significant ($p < 0.05$) increase in inulin associated with the brain parenchyma as compared to Group I. A significant ($p < 0.05$) increase in inulin permeability across the BBB was observed in the cerebral cortex of Group II at 56 (2.3 ± 0.1) and 90 d (2.5 ± 0.2) as compared to Group I (1.8 ± 0.1 and 1.8 ± 0.1 , respectively). Group III showed increased permeability of inulin across the BBB in the cerebral cortex at 90 d (2.4 ± 0.1) as compared to Group I (1.8 ± 0.1). Groups II and III exhibited increased inulin permeability across the BBB in the midbrain at 56 (2.1 ± 0.3 and 1.8 ± 0.1 , respectively) and 90 d (2.3 ± 0.1 and 2.0 ± 0.1 , respectively) as compared to Group I (1.1 ± 0.1 and 1.1 ± 0.1 , respectively). A significant ($p < 0.05$) increase in inulin associated with the brain parenchyma in the basal ganglia was observed in Group II at 56 (1.7 ± 0.01) and 90 d (2.3 ± 0.1) as compared to both Groups I (1.2 ± 0.1 and 1.2 ± 0.1 , respectively) and III (1.3 ± 0.1 and 1.2 ± 0.1 , respectively). A significant ($p < 0.05$) interaction was observed between treatment and day within brain regions.

In situ brain perfusion using [^{14}C] sucrose: BBB permeability was assessed in whole brain of

rats from Groups I, II, and III at 7, 28, and 56 d after STZ-induction by *in situ* brain perfusion with an impermeant marker ($[^{14}\text{C}]$ sucrose; 342 Da) over 20 min (Figure 1C). Results showed no significant difference ($p>0.05$) in sucrose associated with total brain between the treatment groups (II and III) as compared to Group I at 7 d. A significant increase ($p<0.05$) in sucrose associated with total brain of Group II (2.96 ± 0.13) was noted at 28 d as compared to Groups I (2.14 ± 0.02) and III (2.19 ± 0.05). At 56 and 90 d, both Groups II (3.75 ± 0.12 and, 4.73 ± 0.54 , respectively) and III (3.17 ± 0.16 and 3.70 ± 0.20 , respectively) showed a significant ($p<0.05$) increase in sucrose association with total brain as compared to Group I (2.11 ± 0.6 and 2.11 ± 0.6 , respectively). A significant ($p<0.05$) interaction between treatment and day was observed.

Determination of regional differences in $[^{14}\text{C}]$ sucrose association with brain: Using *in situ* brain perfusion with $[^{14}\text{C}]$ sucrose, we assessed rat in Groups I, II, and III for changes in permeability of sucrose into different brain regions at 7, 28, and 56 d following STZ-induced diabetes (Table 3). Results showed a significant ($p<0.05$) increase in BBB permeability to $[^{14}\text{C}]$ sucrose in a number of brain regions of Groups II and III when compared to Group I. A significant ($p<0.05$) increase in sucrose permeability was observed in the cerebral cortex of Group II at 28 (2.5 ± 0.3) and 56 d (4.5 ± 0.3) as compared to both Groups I (2.0 ± 0.2 and 2.0 ± 0.2 , respectively) and III (2.0 ± 0.2 and 2.4 ± 0.2 , respectively). No significant ($p>0.05$) difference was observed between the cortex of Groups I and III at days 7, 28, and 56. At 90 d, Groups II and III demonstrated a significant ($p<0.05$) increase in sucrose permeability in the cerebral cortex as compared to Group I. No significant ($p>0.05$) difference between Groups II and III were observed in the cortex at 90 d. Group II exhibited a significant ($p<0.05$) increase in sucrose associated with the brain parenchyma in the hippocampus at 56 (2.4 ± 0.1) and 90 d (2.8

± 0.2) as compared to Groups I (1.5 ± 0.1 and 1.5 ± 0.1 , respectively) and III (1.7 ± 0.2 and 1.8 ± 0.2 , respectively). At 28, 56, and 90 d, Groups II (3.1 ± 0.3 , 3.5 ± 0.4 , and 4.6 ± 0.5 , respectively) and III (2.1 ± 0.3 , 2.8 ± 0.3 , and 3.7 ± 0.5 , respectively) showed a significant ($p < 0.05$) increase in sucrose permeability across the BBB in the midbrain as compared to Group I (1.4 ± 0.1 , 1.4 ± 0.1 , and 1.4 ± 0.1 , respectively). The difference in the increase of sucrose permeability across the BBB in the midbrain at 28 and 56 d between Groups II and III was significant ($p < 0.05$). At 56 d, Group II (3.6 ± 0.5) showed a significant ($p < 0.05$) increase in sucrose permeability across the BBB in the basal ganglia as compared to both Groups II (2.2 ± 0.3) and III (1.7 ± 0.1). At 90 d, Groups II (3.8 ± 0.5) and III (3.5 ± 0.2) had a significant ($p < 0.05$) increase in sucrose permeability across the BBB in the basal ganglia as compared to Group I (1.7 ± 0.1). A significant ($p < 0.05$) interaction between treatment and day within brain regions was observed.

Determination of sucrose/inulin associated with cerebral microvessels using capillary depletion:

Table 4 shows capillary depletion data in Groups I, II, and III after a 20 min in situ brain perfusion using [^3H]inulin and [^{14}C]sucrose at 7, 28, 56, and 90 d. Capillary depletion data showed no significant ($p > 0.05$) difference in the amount of inulin or sucrose trapped in the vascular pellet between the groups at any day evaluated. Furthermore, the study revealed that the percent amount of inulin/sucrose associated with actual entry into the brain parenchyma (supernatant) was not statistically ($p > 0.05$) different from that in total brain homogenate.

Measurement of cerebral blood flow: Table 5 shows parameters measured for CBF after a 10 s in situ perfusion. Cerebral perfusion pressures and rates showed no difference between Groups II

and III as compared with control (Group I) at 7, 28, 56, and 90 d. Cerebral blood flow (F_{bl}) was calculated at $t = 10$ s using in situ brain perfusion with [3H] butanol. Results showed no significant ($p > 0.05$) change in CBF in the treatment groups (Groups II and III) as compared with control (Group I). Brain weights and the percent water content were similar among all treatment groups (Groups II and III) compared with control (Group I).

2.5 DISCUSSION

The main findings of this study were that STZ-induced diabetes produced a progressive increase in BBB permeability to small molecules from 28 to 90 d and these changes in BBB permeability were region specific with the midbrain seemingly most susceptible to diabetes-induced microvascular damage. Furthermore, this study showed that increased association of a vascular space marker with the brain parenchyma was due to transfer from the systemic circulation to the brain and not increased trapping or endocytosis into brain capillary endothelial cells. In addition, this study showed that insulin treatment of diabetes attenuated BBB disruption, especially during the first few weeks; however, as diabetes progressed, it was evident that microvascular damage occurred even when hyperglycemia was controlled. Finally, this study demonstrated that STZ-induced diabetes did not alter total cerebral blood flow or edema formation at any time point evaluated; therefore, it is improbable that these factors played a role in the increased association of radiolabeled vascular space marker with the brain.

STZ induced hyperglycemia in >95% of rats by 24 h after administration. Availability of 10% glucose water was used to alleviate potential severe hypoglycemia in the rats as a result of increased insulin release during the destruction of pancreatic beta cells. To limit episodes of hypoglycemia in Group III animals, insulin treatment was divided into two doses and glucose levels were regularly monitored. Using this approach, Group III animals had a fasting blood glucose level of 128 ± 19 mg/dl as compared to 146 ± 10 mg/dl in Group I and 426 ± 31 mg/dl in Group II animals.

Changes in BBB permeability due to STZ-induced diabetes were assessed using three varying sized vascular space markers [albumin (65,000 Da), inulin (5,000 Da), sucrose (342 Da)]. Moreover, using differential permeability of radiolabeled vascular space markers has been

effective at measuring regional changes in brain uptake (Pan et al., 1997;Pan et al., 1996;Zlokovic et al., 1986). Assessment of albumin extravasation is a frequently used method to measure vascular leakage. The intact BBB has negligible transport of albumin into the brain and serves as a physical barrier to partition the systemic circulation from the brain parenchyma. However, susceptibility of the BBB to increased permeability has been documented in a number of diseases. While diabetic-induced microvascular leakage is commonly associated with early endothelial dysfunction of the retina (Cukiernik et al., 2004), kidney (Casey et al., 2005), and peritoneum (Wong et al., 2003); few studies have documented changes in the brain. One possible reason for this may be due to BBB phenotype and the relative resistance of the BBB to damage as compared to other microvascular areas. In this study, we measured albumin extravasation into the brain parenchyma by quantifying the amount of Evan's blue albumin extracted from the brains of rats in Groups I, II, and III. No change in total albumin extravasation was observed in the treatment groups (II and III) as compared to control rats; thus, suggesting that the BBB remained intact. This observation was reinforced by measurements showing no change in % brain water, a hallmark indicator of increased extracellular albumin and edema formation. While total albumin extravasation remained unchanged, upon closer evaluation of brain regions, we noted a significant increase in Evan's blue albumin in the midbrain of Groups II and III at 90 d, and in the basal ganglia of Group II at 90 d. These results suggest that the BBB is not a homogenous membrane but rather may have areas of increased susceptibility. Moreover, these results argue in favor of our rationale that by the time albumin is measured, the BBB is compromised and therefore assessment of smaller vascular markers would be more indicative of how the BBB is responding to diabetes-related vascular complications.

In situ brain perfusion, which was used to measure changes in BBB permeability to inulin

and sucrose, is a methodology that has successfully assessed drug transport across the BBB into the CNS (Egleton et al., 2003;Hau et al., 2002;Koziara et al., 2003) and, more recently, changes in BBB function following pathology (Egleton et al., 2003;Hawkins et al., 2004;Huber et al., 2002;Witt et al., 2003). *In situ* brain perfusion has been shown to maintain good neurological function and other physiological factors in rat and guinea pig models (Preston et al., 1995;Zlokovic et al., 1986) and when coupled to capillary depletion, these techniques are precise tools for assessing BBB function. Using these techniques, we determined that BBB functional integrity was maintained to molecules >5000 Da out to 90 d of STZ-induced diabetes; however, BBB functional integrity was disrupted to small molecules by 28 d and progressively worsened out to 90 d. In addition, our studies indicated that control of STZ-induced diabetes with insulin treatment attenuated BBB disruption to sucrose. However, as diabetes progressed, insulin treatment in Group III did not effectively reduce the increased BBB permeability to sucrose observed at 56 and 90 d. The finding that BBB disruptions increased over time supports our rationale that effects of diabetes on the cerebral microvasculature occur in a more insidious manner than other microvascular deficits. Furthermore, since diabetes is a life-long disease, occurring even more prevalently in younger populations, the long term effects of diabetes on brain function are of paramount importance.

We assessed whether diabetes-induced BBB permeability to inulin and sucrose were occurring globally or in specific brain regions. Early changes in microvascular permeability noted in other tissues were not apparent in the BBB even to small molecules. Although no change in inulin permeability was observed in the total brain at any time point assessed, when brain regions were evaluated separately we observed increased permeability to inulin in the cerebral cortex and midbrain in Group II at 56 and 90 d, in the basal ganglia of Group II at 90 d,

and in the midbrain of Group III at 90 d. Assessment of brain region specific permeability of sucrose demonstrated a greater distribution of sucrose than observed with inulin. We observed increased permeability in the cortex at 28, 56, and 90 d in Group II and increased permeability in the midbrain, hippocampus, and basal ganglia in Group II at 56 and 90 d. Group III showed an increased permeability in the midbrain at 56 and 90 d and in the cerebral cortex and basal ganglia at 90 d. These findings suggest that the BBB has certain areas more susceptible to breakdown than others. Based upon our findings and the current literature, we hypothesize that BBB disruptions observed in this study were due to changes in cell-cell contacts leading to increased paracellular flux; however, the possibility exists that these changes were due to alterations in transcytotic pathways or increased capillary fenestrations and future studies will need to address this important issue.

Findings that BBB permeability changes were regional rather than global are not surprising and yet this area has been understudied in regards to the effects of diabetes. Cerebral blood flow and capillary density are not evenly distributed in white and grey matter areas of the brain (Baborie and Kuschinsky, 2006). Under basal conditions, areas with higher metabolic need (i.e. greater demand for glucose) have greater capillary density and increased cerebral blood flow. However, during CNS pathology, the area of the brain affected has a large influence on the ability for recovery. Results from this study suggest a differential susceptibility to diabetes-induced BBB disruption in brain regions. BBB disruptions in the midbrain, an area with lower capillary density and cerebral blood flow than the cortex, were observed at 28 d and were larger in size than seen in other brain areas. Clinical case reports cite an increased susceptibility to third nerve palsies in diabetics due to an increased prevalence of midbrain lesions and hemorrhaging (Heckmann et al., 2003; Mizushima and Seki, 2002). Furthermore, diabetic-

induced lesions have been reported to attenuate morphine analgesia due to decreased serotonergic activity in the raphe magnus nucleus (Ramakrishnan et al., 2004; Sounvoravong et al., 2004). Of particular interest in this investigation was the finding that other brain areas with greater cerebral flow were affected as diabetes progressed to 56 and 90 d. These findings suggest that diabetes-related changes to the central nervous system (i.e. increased oxidative stress, decreased vascular reactivity, altered access to metabolic substrates) have a cumulative effect that takes a greater period of time to manifest in altered BBB function.

The “neurovascular unit”, which is composed of cerebral endothelial cells, pericytes, glia, and neurons, carefully orchestrates localized changes in cerebral blood flow to rapidly meet metabolic demands. Under basal physiological conditions, the spatial and temporal relationship between neural activity and cerebral blood flow, termed neurovascular coupling, utilizes cerebrovascular changes induced by activation to map regional changes in function in the human brain (Girouard and Iadecola, 2006; Koehler et al., 2006). Moreover, cerebral blood flow is maintained in a narrow range by autoregulatory mechanisms, irrespective of changes in systemic blood flow (Rosengarten et al., 2001). However, in several brain pathologies, interactions between neural activity and cerebral blood vessels are disrupted, and the resulting homeostatic unbalance, known as neurovascular uncoupling, may contribute to brain dysfunction, including but not limited to BBB disruptions. In Alzheimer’s disease, hypertension, and ischemic stroke, cerebrovascular function is altered, resulting in reduced cerebral blood flow, altered autoregulation, disruption in nutrient trafficking across the BBB, and attenuated response to increased metabolic demand (Boado, 1998; Girouard and Iadecola, 2006; Mooradian, 1988; Parihar and Pandit, 2003). Often these changes in cerebrovascular function precede onset of any cognitive impairment, suggesting a role for neurovascular uncoupling in the etiology and

progression of cognitive dysfunction. Using these concepts, we argue that observed regional changes in BBB function were due to a number of factors (differential neuronal viability, increased metabolic demand, oxidative stress) in specific brain regions brought about by diabetes-associated effects, including hyperglycemia, dyslipidemia, and increased cholesterol (Egleton et al., 2001;Faraci, 2005). Future studies would be well served to gain a better understanding of how neural and glia cells respond to diabetes and how changes in these cells affect cerebrovascular function.

Cerebral blood flow in control rats (Group I) using [³H] butanol was consistent with previously reported values ranging from 0.8-1.49 ml/min.g (Table 5) (Huber et al., 2001). The current study showed no change in cerebral blood flow at any time point assessed regardless of treatment. A number of factors associated with diabetes have been found to play an integral role in cerebrovascular changes, including oxidative stress, hyperglycemia, atherosclerosis, and autonomic dysfunction (Baird et al., 2002;Hachinski et al., 1992;Prasad, 2000;Pricci et al., 2003). Results of this study suggest cerebral autoregulation of blood flow in the whole brain remained intact during diabetes, which further reaffirms that the increased association of the vascular space marker with the brain parenchyma was due to changes in BBB functional integrity and not changes in hemodynamics. However, what cannot be extrapolated from our findings are possible regional breakdowns in cerebral autoregulation (e.g. neurovascular uncoupling) and the cerebral vascular response to further stressors, such as an acute hypertensive state or additional oxidative stress in the STZ-treated groups (Groups I and II). Future studies will evaluate the ability of cerebral microvessels to adapt and respond to stressors in diabetic rats.

In summary, we have shown that as diabetes progresses, the extent of microvascular

leakage to small molecules increases. These results suggest that diabetes-induced perturbations to cerebral microvessels may disrupt homeostasis and contribute to long term cognitive and functional deficits. The BBB perturbations observed are to small molecules; nonetheless these disruptions are significant, as noted in the gadolinium-DTPA study (Starr et al., 2003), due to the correlation between increased BBB permeability, altered CNS homeostasis and impaired neuronal function. Interestingly, changes in BBB permeability were region specific and many of the areas affected can be associated with detrimental CNS outcomes, including the midbrain, basal ganglia, cortex, and hippocampus. Thus, these results put forward the rationale that microangiopathy of the cerebrovasculature may play a primary role in the etiology of diabetes-induced CNS disorders. Further studies will evaluate the role of BBB endothelial cell tight junction regulation and basement membrane alterations in increased microvascular permeability and focus on how alterations in neurovascular unit function in the identified brain regions relate to the etiology of adverse CNS effects.

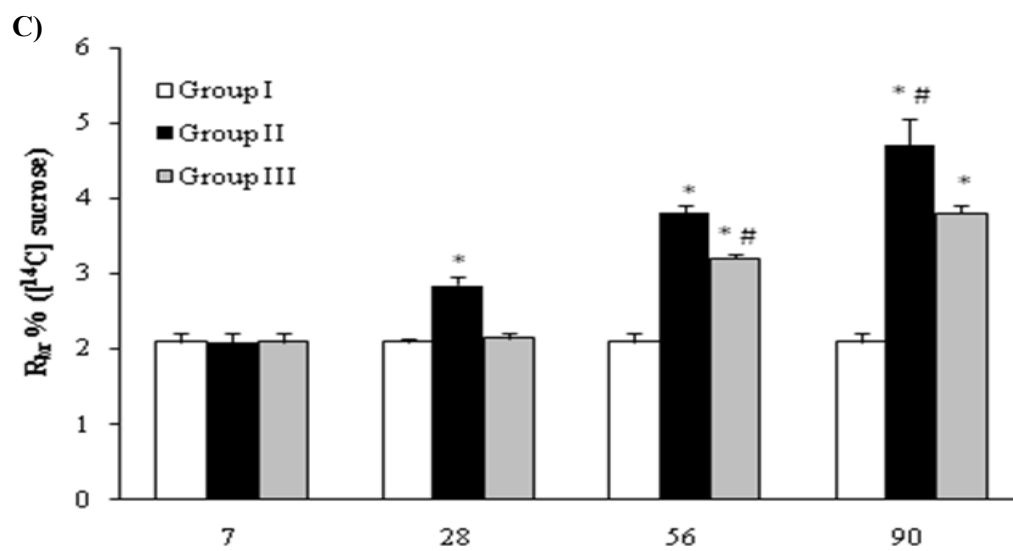
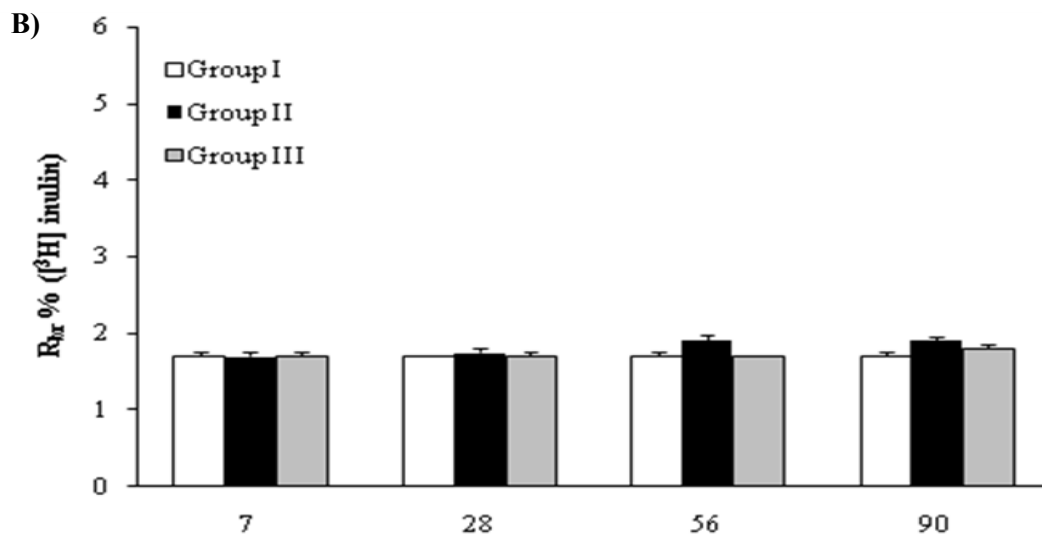
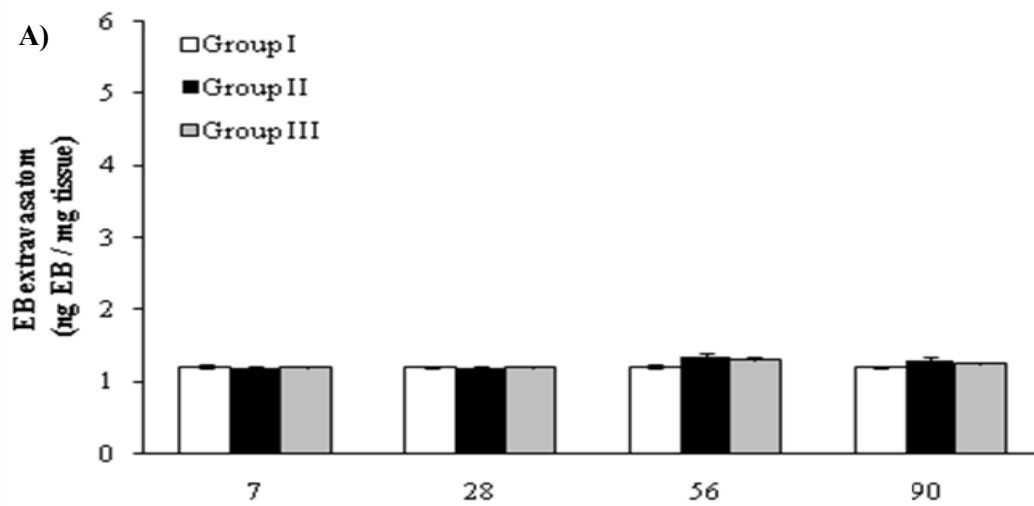


Figure 2.1. Measurement of changes in BBB permeability to vascular space markers in total brain during the progression of streptozotocin-induced diabetes in rats (n=6). Varying sized vascular space markers (A.) albumin, (B.) inulin, and (C.) sucrose were quantified at 7, 28, 56, and 90 d after initial determination of hyperglycemia (Group II). Control animals (Group I) received a single injection (i.p.) of the vehicle and Group III was determined to be hyperglycemic and treated twice daily with regular insulin (4U/d). Bars represent mean \pm S.E. *denotes significant ($P < 0.05$) difference compared with control (*group I*) within day. # denotes significant ($P < 0.05$) difference compared with diabetes (*group II*) within day.

	Day			
	7	28	56	90
Cortex				
Control	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Diabetes	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
Diabetes + insulin	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Cerebellum				
Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Diabetes	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Diabetes + insulin	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Thalamus				
Control	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	1.2 ± 0.1
Diabetes	1.1 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
Diabetes + insulin	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
Midbrain				
Control	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Diabetes	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	1.0 ± 0.2*
Diabetes + insulin	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.9 ± 0.1*
Hypothalamus				
Control	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Diabetes	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Diabetes + insulin	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Hippocampus				
Control	0.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.3	0.8 ± 0.1
Diabetes	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1
Diabetes + insulin	0.9 ± 0.2	0.9 ± 0.3	1.0 ± 0.1	0.9 ± 0.1
Basal ganglia				
Control	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1
Diabetes	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.5 ± 0.2*
Diabetes + insulin	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.3	1.3 ± 0.3

Table 2.1. Measurement of albumin extravasation in various brain regions at 7, 28, 56, and 90 days following STZ-induced diabetes. Values are means \pm SE (in ng Evans blue/mg tissue) for n = 6 rats. Statistical significance was determined using two-way ANOVA followed by Tukey's honest significant difference (HSD) post hoc analyses. STZ, streptozotocin. *Significant ($P < 0.05$) difference compared with control (*group I*) within brain region.

Day	Day			
	7	28	56	90
Cortex				
Control	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Diabetes	1.9 ± 0.1	1.9 ± 0.1	2.3 ± 0.1*†	2.5 ± 0.2*
Diabetes + insulin	1.8 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	2.4 ± 0.1*
Cerebellum				
Control	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Diabetes	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Diabetes + insulin	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Thalamus				
Control	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Diabetes	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
Diabetes + insulin	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.3
Midbrain				
Control	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Diabetes	1.1 ± 0.1	1.2 ± 0.2	2.1 ± 0.3*	2.3 ± 0.1*
Diabetes + insulin	1.1 ± 0.1	1.2 ± 0.1	1.8 ± 0.1*	2.0 ± 0.1*
Hypothalamus				
Control	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Diabetes	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Diabetes + insulin	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
Hippocampus				
Control	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Diabetes	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Diabetes + insulin	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Basal ganglia				
Control	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Diabetes	1.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	2.3 ± 0.1*†
Diabetes + insulin	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1

Table 2.2. $R_{br}\%$ following a 20-min in situ brain perfusion using [^3H]inulin in various brain regions at 7, 28, 56, and 90 days following STZ-induced diabetes. Values are means \pm SE (in $R_{br}\%$ [^3H]inulin) for $n = 6$ rats. Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc analyses. *Significant ($P < 0.05$) difference compared with *group I* (control) within brain region; †significant ($P < 0.05$) difference in percentage of the amount of radioactivity found in the brain compared with that found in the perfusate media ($R_{br}\%$) compared with *group III* (diabetes + insulin) within brain region.

Day	Day			
	7	28	56	90
Cortex				
Control	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2
Diabetes	2.1 ± 0.1	2.5 ± 0.3	4.5 ± 0.3*†	4.3 ± 0.6*†
Diabetes + insulin	2.0 ± 0.2	2.0 ± 0.2	2.4 ± 0.2	3.7 ± 0.3*†
Cerebellum				
Control	2.3 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
Diabetes	2.3 ± 0.1	2.4 ± 0.2	2.5 ± 0.2	2.5 ± 0.3
Diabetes + insulin	2.3 ± 0.1	2.5 ± 0.3	2.5 ± 0.2	2.5 ± 0.3
Thalamus				
Control	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Diabetes	1.8 ± 0.1	2.0 ± 0.2	1.8 ± 0.1	1.9 ± 0.1
Diabetes + insulin	1.8 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.1 ± 0.2
Midbrain				
Control	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Diabetes	1.4 ± 0.1	3.1 ± 0.3*†	3.5 ± 0.4*	4.6 ± 0.5*
Diabetes + insulin	1.4 ± 0.1	2.1 ± 0.3*†	2.8 ± 0.3*	3.7 ± 0.5*
Hypothalamus				
Control	2.5 ± 0.1	2.4 ± 0.2	2.4 ± 0.2	2.5 ± 0.1
Diabetes	2.5 ± 0.3	2.5 ± 0.2	2.6 ± 0.1	2.8 ± 0.4
Diabetes + insulin	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.2	2.7 ± 0.4
Hippocampus				
Control	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
Diabetes	1.5 ± 0.1	1.5 ± 0.1	2.4 ± 0.1*†	2.8 ± 0.2*†
Diabetes + insulin	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.8 ± 0.2
Basal ganglia				
Control	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Diabetes	1.8 ± 0.1	2.1 ± 0.2	3.6 ± 0.5*†	3.8 ± 0.5*†
Diabetes + insulin	1.8 ± 0.1	1.8 ± 0.1	2.2 ± 0.3	3.5 ± 0.2*†

Table 2.3. $R_{br}\%$ following a 20-min in situ brain perfusion using [^{14}C]sucrose in various brain regions at 7, 28, 56, and 90 days following STZ-induced diabetes. Values are means \pm SE (in $R_{br}\%$ [^{14}C]sucrose) for $n = 6$ rats. Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc analyses. *Significant ($P < 0.05$) difference compared with *group I* (control) within brain region; †significant ($P < 0.05$) difference in $R_{br}\%$ compared with *group III* (diabetes + insulin) within brain region.

Day	Parameter	Fraction		
		Pellet	Supernatant	Homogenate
7	Sucrose			
	<i>Group I</i>	0.3 ± 0.2*	1.5 ± 0.3	1.6 ± 0.2
	<i>Group II</i>	0.2 ± 0.1*	1.5 ± 0.2	1.5 ± 0.2
	<i>Group III</i>	0.3 ± 0.2*	1.6 ± 0.3	1.5 ± 0.2
	Inulin			
	<i>Group I</i>	0.2 ± 0.1*	1.4 ± 0.3	1.4 ± 0.2
	<i>Group II</i>	0.2 ± 0.1*	1.4 ± 0.1	1.5 ± 0.1
	<i>Group III</i>	0.2 ± 0.1*	1.5 ± 0.2	1.4 ± 0.2
	28	Sucrose		
<i>Group I</i>		0.3 ± 0.1*	1.4 ± 0.1	1.5 ± 0.2
<i>Group II</i>		0.3 ± 0.1*	2.7 ± 0.2†‡	2.8 ± 0.3†‡
<i>Group III</i>		0.3 ± 0.1*	1.6 ± 0.1	1.6 ± 0.2
Inulin				
<i>Group I</i>		0.2 ± 0.1*	1.4 ± 0.2	1.3 ± 0.1
<i>Group II</i>		0.2 ± 0.1*	1.5 ± 0.2	1.4 ± 0.1
<i>Group III</i>		0.2 ± 0.1*	1.7 ± 0.2	1.4 ± 0.1
56		Sucrose		
	<i>Group I</i>	0.3 ± 0.2*	1.5 ± 0.2	1.4 ± 0.3
	<i>Group II</i>	0.3 ± 0.1*	3.4 ± 0.5†‡	3.6 ± 0.3†
	<i>Group III</i>	0.3 ± 0.2*	1.7 ± 0.4	3.3 ± 0.4†
	Inulin			
	<i>Group I</i>	0.2 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1
	<i>Group II</i>	0.2 ± 0.1*	1.4 ± 0.1	1.6 ± 0.1
	<i>Group III</i>	0.2 ± 0.1*	1.4 ± 0.1	1.2 ± 0.2
	90	Sucrose		
<i>Group I</i>		0.3 ± 0.1*	1.3 ± 0.2	1.5 ± 0.3
<i>Group II</i>		0.3 ± 0.1*	3.7 ± 0.6†	3.9 ± 0.7†
<i>Group III</i>		0.3 ± 0.2*	3.6 ± 0.1†	3.5 ± 0.4†
Inulin				
<i>Group I</i>		0.2 ± 0.1*	1.4 ± 0.1	1.6 ± 0.3
<i>Group II</i>		0.2 ± 0.1*	1.4 ± 0.2	1.4 ± 0.1
<i>Group III</i>		0.2 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1

Table 2.4. Capillary depletion studies after a 20-min in situ brain perfusion. Values are means \pm SE (in Rbr% [^3H]inulin or [^{14}C]sucrose) for $n = 6$ rats. Data are the percent values of sucrose/inulin associated with the brain separated into fractions (vascular pellet and supernatant) and the total brain homogenate. Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc analyses. *Significant ($P < 0.05$) difference from homogenate within treatment group; †significant ($P < 0.05$) difference from *group I* (control) within day and vascular space marker; ‡significant ($P < 0.05$) difference from *group III* (diabetes + insulin) within day and vascular space marker.

Day	Parameter	Group I	Group II	Group III
7	λ_{br}	0.72	0.75	0.74
	Perfusion pressure, mmHg	83.4 ± 4.8	82.8 ± 3.1	83.8 ± 2.4
	Perfusion rate, ml·min ⁻¹ ·g ⁻¹	1.7 ± 0.03	1.69 ± 0.07	1.73 ± 0.02
	Cerebral blood flow, ml·min ⁻¹ ·g ⁻¹	1.22 ± 0.31	1.35 ± 0.21	1.28 ± 0.2
	Perfused brain weight, g	2.1 ± 0.2	2.2 ± 0.2	2.1 ± 0.2
	Perfused brain water, %	82.8 ± 0.4	82.8 ± 0.4	82.7 ± 0.7
28	λ_{br}	0.69	0.74	0.73
	Perfusion pressure, mmHg	81.5 ± 3.6	84.2 ± 4.7	83.0 ± 2.1
	Perfusion rate, ml·min ⁻¹ ·g ⁻¹	1.66 ± 0.04	1.74 ± 0.06	1.71 ± 0.02
	Cerebral blood flow, ml·min ⁻¹ ·g ⁻¹	1.15 ± 0.24	1.26 ± 0.34	1.20 ± 0.12
	Perfused brain weight, g	202 ± 0.3	2.2 ± 0.2	2.2 ± 0.2
	Perfused brain water, %	82.4 ± 1.2	82.7 ± 0.8	83.2 ± 0.9
56	λ_{br}	0.71	0.8	0.75
	Perfusion pressure, mmHg	83.4 ± 2.8	81.7 ± 3.7	86.2 ± 3.3
	Perfusion rate, ml·min ⁻¹ ·g ⁻¹	1.71 ± 0.05	1.76 ± 0.04	1.68 ± 0.05
	Cerebral blood flow, ml·min ⁻¹ ·g ⁻¹	1.31 ± 0.25	1.27 ± 0.19	1.22 ± 0.29
	Perfused brain weight, g	2.2 ± 0.1	2.2 ± 0.2	2.3 ± 0.2
	Perfused brain water, %	81.4 ± 0.8	81.2 ± 1.1	82.4 ± 0.7
90	λ_{br}	0.73	0.74	0.73
	Perfusion pressure, mmHg	87.1 ± 3.6	83.4 ± 4.7	85.5 ± 3.9
	Perfusion rate, ml·min ⁻¹ ·g ⁻¹	1.72 ± 0.04	1.78 ± 0.09	1.67 ± 0.03
	Cerebral blood flow, ml·min ⁻¹ ·g ⁻¹	1.14 ± 0.26	1.34 ± 0.19	1.26 ± 0.21
	Perfused brain weight, g	2.2 ± 0.2	2.2 ± 0.1	2.2 ± 0.2
	Perfused brain water, %	81.1 ± 0.4	81.3 ± 0.6	81.7 ± 1.2

Table 2.5. Cerebral blood flow analyses using in situ brain perfusion with [³H] butanol and measurement of percent brain water in rats at 7, 28, 56, and 90 days following STZ-induced diabetes. Values are means \pm SE for $n = 6$ rats. *Group I*, control; *group II*, diabetes; *group III*, diabetes + insulin; λ_{br} , partition coefficient.

CHAPTER THREE

Administration of sesamol improved blood-brain barrier function in
streptozotocin-induced diabetes rats

This chapter is identical to a manuscript accepted into Experimental Brain Research in April

2008.

3.1 ABSTRACT

Uncontrolled or poorly controlled blood glucose during diabetes is an important factor in worsened vascular function. While evidence suggests that hyperglycemia-induced oxidative stress plays a prominent role in development of microangiopathy of the retina, kidney, and nerves, the role oxidative stress plays on blood-brain barrier (BBB) function and structure has lagged behind. In this study, a natural antioxidant, sesamol, was administered to streptozotocin (STZ)-induced diabetic rats to examine the role that oxidative stress plays on BBB structure and function. Experiments were conducted at 56 d after STZ injection. Male Sprague-Dawley rats randomly were divided into four treatment groups (CON- control; STZ- STZ-induced diabetes; CON+S- control+sesamol; STZ+S- STZ-induced diabetes+sesamol). Functional and structural changes to the BBB were measured by in situ brain perfusion and western blot analysis of changes in tight junction protein expression. Oxidative stress markers were visualized by fluorescent confocal microscopy and assayed by spectrophotometric analysis. Results demonstrated that the increased BBB permeability observed in STZ-induced diabetic rats was attenuated in STZ+S rats to levels observed in CON. Sesamol treatment reduced the negative impact of STZ-induced diabetes on tight junction protein expression in isolated cerebral microvessels. Oxidative stress markers were elevated in STZ as compared to CON. STZ+S displayed an improved antioxidant capacity which led to a reduced expression of superoxide and peroxynitrite. In conclusion, this study showed that sesamol treatment enhanced antioxidant capacity of the diabetic brain and led to decreased perturbation of hyperglycemia-induced changes in BBB structure and function.

3.2 INTRODUCTION

Many of the debilitating consequences associated with diabetes result from prolonged vascular dysfunction. Uncontrolled or poorly controlled blood glucose during diabetes is an important factor in worsened vascular function (The ADVANCE Collaborative Group, 2008). Evidence suggests that hyperglycemia-induced oxidative stress plays a primary role in the vascular complications most commonly associated with diabetes (e.g. retinopathy, nephropathy, peripheral neuropathies) (Figuroa-Romero et al., 2008;Forbes et al., 2008;Kowluru and Kanwar, 2009;Kowluru and Chan, 2007). Oxidative stress results from an imbalance between generation of reactive oxygen species (ROS) and antioxidant capacity. While ROS at low concentrations play important physiological roles as signaling molecules and contribute to localized regulation of vascular tone (Lee and Griendling, 2008;Valko et al., 2007), excess ROS has a number of detrimental implications on vascular function, including depletion of nitric oxide bioavailability, increased nitrosative stress, vascular remodeling, depletion of antioxidant enzymes, and impaired vascular coupling (Valko et al., 2007).

While diabetes-induced effects on large and small peripheral vessels have been well documented (Valko et al., 2007), the role that oxidative stress plays on function and structure of the cerebrovasculature has lagged behind. The lack of studies regarding the effects of diabetes on the brain is surprising considering that people with diabetes have a higher incidence of lacunar infarct, ischemic stroke, and vascular dementia (Appelros et al., 2005;Karapanayiotides et al., 2004;Colwell, 2000). Moreover, people with diabetes are at higher risk of developing cognitive disorders and many researchers regard diabetes as a predisposing factor for Alzheimer's disease (Ristow, 2004). Previously, we reported a time-dependent and

brain region specific increase in blood-brain barrier (BBB) permeability during streptozotocin (STZ)-induced diabetes in rats (Huber et al., 2006). Furthermore, we demonstrated that blood glucose control with insulin only partially attenuated the cerebrovascular damage, thus suggesting that vascular dysfunction had both glucose-dependent and –independent aspects (Huber et al., 2006). Hyperglycemia-induced ROS may contribute to dysregulation of the BBB, which could disrupt the neuronal microenvironment and translate to cognitive deficits.

A recent study found that sesamol, a natural antioxidant, improved glucose regulation, reduced lipid peroxidation in the cortex and hippocampus, and improved cognitive ability in STZ-induced diabetic rats (Kuhad and Chopra, 2008); thus suggesting a role for oxidative stress as a causal factor in brain dysfunction during diabetes. In the present study, we investigated susceptibility of the BBB to oxidative stress and examined the role of long-term sesamol administration on BBB function and structure during STZ-induced diabetes in rats.

3.3 MATERIALS AND METHODS

Chemicals and Animals. All chemicals used in this study were of molecular biology grade and purchased from Sigma Chemical (St. Louis, MO), unless otherwise noted. Male Sprague-Dawley rats (Harlan; Indianapolis, IN) weighing 250–274 g were housed under 12-h:12-h light-dark conditions and received food and water ad libitum. Rats were acclimated for 7 d before induction of diabetes. Weight was determined at 0 and 56 d of the study. All protocols involving rats were approved by the West Virginia University Animal Care and Use Committee and abide by National Institutes of Health guidelines.

Diabetes induction procedures. STZ was dissolved in sodium citrate (50 mM) buffered saline (vehicle). Sesamol was dissolved in 0.9% saline. All injections were given intraperitoneally (i.p.). Rats were randomly divided into four treatment groups. The first treatment group (CON) received an injection of vehicle and then at 7 d post-injection received an injection of saline (200 μ l) daily until 56 d. The next treatment group (STZ) received an injection of STZ (60 mg/kg; 100 μ l) in vehicle and then at 7 d post-injection received an injection of saline (200 μ l) daily until 56 d. The third treatment group (CON+S) received an injection of vehicle and then at 7 d post-injection received an injection of sesamol (10 mg/kg; 200 μ l) daily until 56 d. The final treatment group (STZ+S) received an injection of STZ (100 μ l) in vehicle and then at 7 d post-injection received an injection of sesamol (200 μ l) daily until 56 d. To avoid early mortality from severe hypoglycemia, rats injected with STZ were supplemented with 10% glucose water for 12 h (Huber et al. 2007; Ramachandra et al. 2005; Singh et al. 2005; Cheng et al. 2003; Ramsay and White 2000; Babu and Srinivasan 1999). Glucose levels were checked at 0, 1, 2 (if necessary),

and 56 d. Rats were denoted as having diabetes if blood glucose level measured ≥ 350 mg/dl by 2 d post-STZ injection.

In Situ Brain Perfusion. In situ brain perfusions were carried out based on a previously described method (Preston et al. 1995). Briefly, rats (n=6/group) were anesthetized with an intramuscular injection of rat cocktail [flunixin (2.5 mg/kg), ketamine (90 mg/kg), xylazine (5 mg/kg)], heparinized (10,000 U/kg), and body temperature maintained at 37°C. The right common carotid artery was cannulated and perfused with a modified Krebs-Hanseleit Ringer's solution [117 mM NaCl; 4.7 mM KCl; 0.8 mM MgSO₄; 24.8 mM NaHCO₃; 1.2 mM KH₂PO₄; 2.5 mM CaCl₂; 10 mM D-glucose; 29 g/l dextran (70,000 Da); 10 g/l bovine serum albumin], which was aerated with 95% O₂/5% CO₂ and warmed to 37° C. Once desired perfusion pressure (85-95 mmHg) and flow rate (3.1 ml/min) were achieved for right common carotid artery, the contralateral carotid artery was cannulated and perfused. Once both arteries were cannulated, [¹⁴C]sucrose (New England Nuclear; Waltham, MA) was infused via syringe pump (flow rate: 0.5 ml/min) into the inflowing Ringer's solution (total flow rate: 3.6 ml min⁻¹ hemisphere⁻¹). The jugular veins were sectioned to allow for drainage. After 20 min, brains were flushed for 20 s with unlabeled Ringer's solution. Rats were sacrificed by decapitation, brains removed and choroid plexi and meninges excised. Perfusion fluid (500 µl) was collected from cannulas by briefly resuming perfusion of [¹⁴C]sucrose following termination. Brain tissue samples (500 mg) and 100 µl of perfusate samples were prepared for radioactive counting by addition of 1 ml of tissue solubilizer (Research Products International; Mount Prospect, IL), 30 µl glacial acetic acid (to quench chemiluminescence), and 4 ml scintillation cocktail (Budget Solve; Research Products International). Samples were analyzed by liquid scintillation counting. Amount of [¹⁴C] radioactivity in brain [C_{brain} , disintegrations per minute (dpm) per gram brain (dpm/g)] was

expressed as a percentage of radioactivity in perfusate ($C_{\text{perfusate}}$; dpm/ml) and termed $R_{\text{brain (br)}}\%$ ($\mu\text{l/g}$) as follows: $R_{\text{br}}\% = (C_{\text{brain}}/C_{\text{perfusate}}) \times 100\%$.

Capillary depletion studies. Capillary depletion method was performed as previously described (Triguero et al. 1990). After in situ brain perfusion, brains were removed, choroid plexi and meninges excised, and brains homogenized in 1.5 ml of capillary depletion buffer [15 mM HEPES; 141 mM NaCl; 4 mM KCl; 2.8 mM CaCl_2 ; 1 mM MgSO_4 ; 10 mM D-glucose]. Two aliquots of homogenate were taken and centrifuged at $5,400 \times g$ for 15 min. Capillary-depleted supernatant was separated from vascular pellet. Homogenate, supernatant, and pellet were counted for radioactivity on scintillation counter. All homogenization procedures were carried out within 2 min.

Sample Preparation. At 56 d post-STZ injection, rats were anesthetized with sodium pentobarbital (60 mg/kg; 200 μl) and underwent cardiac perfusion with heparinized saline (2 U/ml) for 5 min via the left ventricle. Perfusion fluid was switched to 10% neutral-buffered formalin (fixative) and perfused for 5 min. After perfusion, rats were sacrificed by decapitation, brains extracted, and placed in fixative overnight at 4°C . Brains were cryopreserved in 20% and 30% sucrose solutions, embedded in Tissue-Tec[®] optimal cutting temperature compound (Miles; Elkhart, IN), sliced into 30 μm sections, mounted on slides, and frozen at -20°C until used.

Determination of superoxide production. Conversion of dihydroethidium (DHE) to ethidine was used as an indirect measurement of superoxide production. DHE was dissolved in saline (0.5 mg/ml). Four rats from each treatment group were injected (i.p.) with DHE (250 μl), which was allowed to circulate for 1 h. After sample preparation (see above), brain sections were coverslipped and viewed at $40\times$ magnification on an LSM 510 confocal microscope (Carl Zeiss;

Thornwood, NY) at 543 nm. All samples were viewed at the same time. All the settings (pinhole, gain, laser intensity) were kept the same between samples.

Detection of peroxynitrite. Peroxynitrite cannot be detected in vivo due to its short half-life; therefore, nitrotyrosine is utilized as a biomarker for changes in peroxynitrite levels. Upon use, slides were rinsed in 1× PBS (3 × 10 min). Brain sections were permeabilized in 1× PBS + 0.1% Triton-X 100 for 10 min. Brain sections were incubated overnight at 4° C in blocking buffer (LI-COR Biosciences; Lincoln, NE) to reduce nonspecific binding. Brain sections were incubated with mouse anti-nitrotyrosine (1:250; Cayman Chemicals; Ann Arbor, MI) in a humidified chamber for 1 h at 37° C and then incubated in a humidified chamber for 30 min at 37° C with fluorescent labeled (FITC) goat anti-mouse IgG (1:1000; Invitrogen; Carlsbad, CA). After rinsing, slides were dried at 70° C, and coverslipped. Images were viewed at 40× magnification on an LSM 510 confocal microscope at 488 nm. All samples were stained at the same time. All settings (pinhole, gain, laser intensity) were kept the same between treatment groups (n=4 rats/group).

Measurement of catalase activity. Catalase activity was measured using a catalase assay kit (Cayman Chemicals). Briefly, 50 mg brain tissue (n=6 rats/group) was homogenized 1:5 in 50 mM KH₂PO₄ (pH 7.0) containing 1 mM EDTA. The assay was carried out according to protocol and absorbance was read at 540 nm.

Microvessel isolation. At 56 d, rats (n=3/group) were anesthetized with sodium pentobarbital (60 mg/kg; i.p.), sacrificed by decapitation, and brains removed. Choroid plexi and meninges were excised and cerebral hemispheres homogenized in 4 ml of microvessel isolation buffer [103 mM NaCl; 4.7mM KCl; 2.5 mM KH₂PO₄; 1.2 mM MgSO₄; 15 mM HEPES; 2.5 mM NaHCO₃;

10 mM D-glucose; 1 mM Na pyruvate; 10 g/l dextran (64,000 Da)] with protease inhibitors (Roche; Indianapolis, IN). Four milliliters of 26% dextran were added to homogenate and vortexed. Homogenates were centrifuged at 5,600 x g for 10 min and supernatant aspirated. Pellets were resuspended in 10 ml of microvessel isolation buffer, passed through a 70 µm filter, and centrifuged at 3,000 x g.

Determination of changes in tight junction protein expression. Total protein from isolated cerebral microvessels (n=3 rats/group) was isolated and resuspended in 250 µl of boiling 1× SDS and heated at 90°C. After 30 min, homogenates were centrifuged at 12,000 x g for 10 min at 4°C. A 20 µl aliquot was taken for protein determination using a bicinchoninic acid protein assay (Pierce; Rockford, IL) with bovine serum albumin as the standard. The remaining supernatant was transferred to a new microfuge tube and stored at -80°C until needed. Proteins (10 µg) were electrophoretically resolved and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated in primary antibody [rabbit anti-ZO 1 (1:1,000; Invitrogen); rabbit anti claudin 5 (1:1,000; Invitrogen); rabbit anti-occludin (1:2,000; Invitrogen); mouse anti-β actin (1:2,500)]. After 1 h, membranes were incubated in peroxidase labeled secondary antibody [anti-rabbit IgG (1:2,000) and anti-mouse IgG (1:5,000)] for 30 min and then developed by chemiluminescence and analyzed with Scion Image software (Scion; Frederick, MD). Proper protein loading was confirmed using GelCode[®] Blue (Pierce) and tight junction protein expression was normalized to β-actin and expressed as %CON expression.

Determination of changes in tight junction mRNA expression. Total RNA was isolated from cerebral microvessels (n=3 rats/group). Concentration of RNA was determined and only considered for use if A_{260}/A_{280} was ≥ 1.80 . Total RNA was reverse transcribed into cDNA using a 20 µl reaction. Real time PCR analyses of claudin 5, occludin, and zo-1 using a StepOne™

detection system (Applied Biosystems; Foster City, CA) in combination with TaqMan[®] chemistry. Specific primers and dual labeled internal (FAM/TAMRA) probe sets were used according to manufacturer's recommendation (Applied Biosystems). All PCR amplifications were run in a 20 μ l reaction volume consisting of 1 μ l cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control to normalize for differences in amount of cDNA added to reactions. Negative controls were monitored simultaneously within each run. Thermocycling conditions were set to holding stage at 50° C for 2 min and 95°C for 10 min and 40 cycles of 95° C for 15 s and 60° C for 1 min. According to manufacturer's instructions, relative quantification of mRNA transcripts was carried out using the comparative threshold ($\Delta\Delta C_T$) method.

Statistical Analysis. Data were expressed as mean \pm S.E.M. Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test. Level of significance was set at $p < 0.05$.

3.4 RESULTS

Physiological Outcomes. Plasma glucose (n=18 rats/group), glycosylated hemoglobin (Hb_{A1c}) levels (n=6 rats/group), and change in body weight (n=18 rats/group) were measured at 56 d (Table 3.1). Mortality rates over the 56 d were CON (0/18), STZ (0/18), CON+S (0/18), and STZ+S (1/19).

In situ brain perfusion and capillary depletion studies. BBB permeability (n=6 rats/group) was measured at 56 d (Figure 3.1). Results demonstrated a significant increase in BBB permeability in STZ (4.0±0.1) as compared to CON (1.7±0.1). Administration of sesamol decreased BBB permeability in STZ+S as compared to STZ (1.7±0.2 and 4.0±0.1, respectively). No difference in BBB permeability to [¹⁴C]sucrose between CON, CON+S and STZ+S was observed.

Capillary depletion data showed no difference in amount of sucrose associated with the vascular pellet between treatment groups. Furthermore, the study revealed that percent amount of sucrose associated with actual entry into the brain parenchyma (supernatant) was not different from that in total brain homogenate (data not shown).

Determination of superoxide production. Superoxide production (n=4 rats/group) was visualized in brain regions at 56 d using confocal microscopy (Figure 3.2). Figure 4a and 4b are representative negative controls for DHE staining in CON and STZ. Other brain regions including striatum (Figure 3.4c and 3.4d), thalamus, hippocampus, and cerebellum showed no observable difference in superoxide production in STZ as compared to CON.

Detection of peroxynitrite production. Nitrotyrosine immunoreactivity (n=4 rats/group) was visualized in brain regions at 56 d using confocal microscopy (Figure 3.3). Figure 3.4e and 3.4f

are representative negative controls for antinitrotyrosine staining in CON and STZ. No observable difference in antinitrotyrosine staining was observed between CON and STZ in the striatum (Figure 3.4g and 3.4h), thalamus, hippocampus, or cerebellum.

Measurement of catalase activity. Catalase activity (n=6 rats/group) in the brain was measured at 56 d (Figure 3.5). Results showed that CON+S and STZ+S had significantly increased catalase activity as compared to CON and STZ. No difference was observed between CON and STZ.

Determination of changes in tight junction protein expression. Changes in tight junction protein expression in isolated cerebral microvessels were assessed by western blot and densitometric analysis (n=3 rats/group) at 56 d. Tight junction protein expression was normalized to β -actin and expressed as % CON expression (Figure 5.6). Results indicated a significant effect of sesamol administration between treatment groups for ZO-1 and claudin 5. Presence of diabetes indicated a significant effect in ZO-1, occludin, and claudin 5. STZ showed a significant decrease in claudin 5, occludin, and ZO-1 protein expression as compared to CON and STZ+S. No difference in tight junction protein expression between CON, CON+S, STZ+S was observed.

Determination of changes in tight junction mRNA expression. Real time PCR (n=3 rats/group) was performed to measure changes in gene expression of tight junction proteins at 56 d (Figure 7). Changes in gene expression were calculated based on the $\Delta\Delta C_T$ method with GAPDH serving as the endogenous control. STZ+S showed increased claudin 5 mRNA expression as compared to CON (5.8 fold) and STZ (3.8 fold). Occludin gene expression was decreased in STZ as compared to CON (9.8 fold) and STZ+S (10.6 fold). No change in occludin mRNA

expression was observed between CON and STZ+S. No difference in mRNA expression between CON and CON+S was observed for any tight junction gene.

3.5 DISCUSSION

The major findings of this study were that sesamol treatment enhanced antioxidant capacity of the diabetic brain and prevented increased BBB permeability. Diabetic rats treated with sesamol showed reduced superoxide and peroxynitrite production in the brain in the same regions previously shown to be most susceptible to BBB dysfunction (Huber et al., 2006). The BBB is a heterogeneous structure (Banks et al., 1999), in which the vasculature in certain regions of the brain are more vulnerable to oxidative damage (Lovell and Markesbery, 2007;Cardozo-Pelaez et al., 2000) and neurovascular uncoupling (Osawa et al., 2004); thus our findings suggest that areas of the brain affected by BBB dysregulation may be an indicator of the types of neurological complications associated with long-term diabetes .

Due to destruction of pancreatic beta cells following STZ administration, rats experience an early, uncontrolled release of insulin into the circulation. The resulting hypoglycemia can be profound leading to lethargy, coma, and death. While mortality rates are often not provided, those studies that do, report a mortality rate between 10-20% and some as high as 50% following STZ administration (Marathe et al., 2006;Di Leo et al., 2004;Wellmann and Volk, 1977). Previously, in our lab, we experienced a mortality rate of ~20% at 7 d post STZ administration. Amending our protocol to include supplementation with 10% glucose water for a short period of time after STZ administration has reduced mortality to < 1% during the first 7 d after administration of STZ.

Sesamol treatment had no effect on the decreased weight gain and increased blood glucose levels often seen in STZ-induced diabetic rats (Kuhad and Chopra, 2008;Huber et al., 2006;Chehade et al., 2002;Hawkins et al., 2007;Egletton et al., 2003). These results contrasted

with a previous study (Kuhad and Chopra, 2008), which reported that sesamol treatment significantly reduced blood glucose. A possible explanation for this discrepancy may be the time frame in which sesamol treatment was administered. We began sesamol treatment at 7 d following STZ injection as compared to 3 d in the other study (Kuhad and Chopra, 2008). When STZ is administered, beta cell function in the pancreas is initially impaired by altered glucose oxidation and decreased insulin biosynthesis and secretion (Bedoya et al., 1996; Nukatsuka et al., 1990). This is followed by a temporary glucose response and then oxidative stress driven cell damage and permanent cell loss (West et al., 1996). Excess generation of reactive oxygen and nitrogen species play a primary role in STZ-induced beta cell death in the pancreas (Bedoya et al., 1996; Turk et al., 1993); thus, if an antioxidant, such as sesamol, is given before this process is completed, partial preservation of beta cells can reverse the hyperglycemic effect (Cam et al., 1997; Van et al., 2008). To further confirm our findings that sesamol treatment had no effect on blood glucose regulation, we measured glycosylated hemoglobin (Hb_{A1c}) levels in the blood. Hb_{A1c} , an indicator of average blood glucose levels over an extended period of time, equated to a mean blood glucose level between 60-90 mg/dl in non-diabetic rats; whereas both STZ-induced diabetic rats and the sesamol treated STZ-induced diabetic rats had mean blood glucose levels between 330-360 mg/dl. Thus, confirming that sesamol treatment had no effect on regulating blood glucose levels during diabetes.

Uncontrolled or poorly controlled hyperglycemia causes an imbalance between activity of ROS generating enzymes and antioxidant capacity (Figuerola-Romero et al., 2008; Forbes et al., 2008; Kowluru and Chan, 2007). Elevated blood glucose levels lead to decreased nitric oxide bioavailability, which contributes to unmet metabolic demand of neurons (Fouilloux et al., 2008; Kumagai, 1999), impaired neurovascular coupling (Riva et al., 2005) and loss of vascular

reactivity (Oltman et al., 2008). Dysfunction of metabolic pathways in brain vasculature during diabetes has been hypothesized to precede noticeable cognitive deficits (Ryan et al. 2003; Ryan 2005). In a previous study, we showed that STZ-induced diabetes caused a progressive, region specific increase in BBB permeability (Huber et al., 2006). These changes, while not as prominent as that seen in other diseases (Brooks et al., 2005; DiNapoli et al., 2008; Kaya et al., 2008; Tomkins et al., 2008; Zlokovic, 2008; Huber et al., 2006; Huber et al., 2002), suggest that impairment of BBB function during diabetes may be a predisposing factor for the increased incidences of vascular dementia and cerebrovascular disease in people with diabetes (Nelson et al., 2008; Ristow, 2004). The present study demonstrated that STZ-induced diabetes caused a marked elevation in markers of oxidative stress in cerebral microvessels of the cortex, hippocampus, and midbrain. No signs of increased oxidative stress were observed in other brain regions, including the cerebellum and basal ganglia. These findings corroborate previous studies (Celik and Erdogan, 2008; Santos et al., 2001) and suggest that oxidative stress plays a role in the progressive, region specific increase in BBB permeability.

To sustain pro-oxidant/antioxidant balance, antioxidant enzymatic capacity must be maintained. Oxidative stress during diabetes increases the level of hydrogen peroxide by reducing the activity of thiol-containing enzymes (e.g. thioredoxin and glutathione peroxidase) (Kowluru and Chan, 2007). Elevated hydrogen peroxide leads to increased generation of hydroxyl radicals, which can oxidize metal containing enzymes (e.g. superoxide dismutase and catalase). This cycle leads to disruption in the antioxidant enzyme system. Results of this study demonstrate that sesamol treatment increased catalase activity by 47% and reduced the expression of oxidative stress markers in the brain of diabetic rats. While this study suggests that sesamol treatment increased the antioxidant capacity of the brain, the precise mechanisms

involved are still unclear. Using in vitro experiments, sesamol scavenges various ROS, including lipid peroxy radicals, hydroxyl radicals, and superoxide (Aboul-Enein et al., 2007; Joshi et al., 2005). Interestingly, sesamol is a precursor in the production of paroxetine, which has been shown, in addition to its antidepressant effect, to have a therapeutic benefit in treating symptoms of diabetic neuropathy (Sindrup et al., 1990; Sindrup et al., 1991). Structurally, both sesamol and paroxetine have two functional groups (phenolic ring and benzodioxyl group) capable of scavenging ROS. The benzodioxyl group scavenges hydroxyl radicals, which in excess slows oxidative chain reactions such as the Haber-Weiss and Fenton reactions (Aboul-Enein et al., 2007; Joshi et al., 2005). The brain, due to its high lipid content, is particularly susceptible to damage from oxidative chain reactions initiated by hydroxyl radicals (Reiter et al., 1998). Further studies need to clarify the major enzyme systems in this process. Of particular interest are transition-metal containing enzymes, due to their vulnerability to hydroxyl radicals (Reiter et al., 1998).

An unexpected finding in this study was the effectiveness of chronic sesamol to prevent increased BBB permeability during diabetes. What cannot be ascertained from these studies is whether changes in BBB permeability were due to increased paracellular transport or enhanced endocytosis. Several studies report increased endocytosis at the endothelium during diabetes (Popov and Simionescu, 2001; Simionescu and Antohe, 2006). However, results from our capillary depletion study combined with previously reported changes in BBB function during diabetes (Huber et al., 2006; Hawkins et al., 2007) suggest that increased BBB permeability is primarily due to alterations in paracellular transport. Generation of excess ROS has been shown to increase BBB permeability by alteration in tight junction protein expression (Schreibelt et al., 2007) and increased vascular remodeling (Haorah et al., 2008). While our study only focused on

changes in tight junction proteins during STZ-induced diabetes, ROS certainly have a role in altering the basement membrane during diabetes (Balakumar et al., 2009;Manea et al., 2004). In fact, vascular remodeling is a hallmark indicator of diabetes-induced angiopathies with characteristic thickening of the basement membrane and hardening of the blood vessels (Manea et al., 2004). In addition, increased vascular permeability and changes in tight junction protein expression during diabetes are well documented in the retina (Harhaj et al., 2006;Kim et al., 2009). Gaining a better understanding of the molecular mechanisms through which oxidative stress disrupts BBB function and the interrelationship between tight junctions and changes in basement membrane may reveal novel opportunities for treatment of diabetic complications.

Decreased protein expression of ZO-1, occludin, and claudin 5 was measured in isolated cerebral microvessels of STZ-induced diabetic rats. These findings are in agreement with two earlier reports that showed decreased expression of occludin (Hawkins et al., 2007;Chehade et al., 2002) and/or ZO-1 (Hawkins et al., 2007) in STZ-induced diabetic rats; however, our findings contrast with a previous report which found no change in claudin 5 expression. Sesamol treatment eliminated the decreased ZO-1 and claudin 5 protein expression observed in STZ-induced diabetic rats, while no improvement in occludin expression was demonstrated. Interestingly, denatured occludin has been previously shown to migrate as two distinct bands denoted as α and β (Huber et al., 2002;Antonetti et al., 1998). Phosphorylation of occludin regulates tight junction function by redistributing occludin from the cytoplasm to the lateral surface of the plasma membrane (Andreeva et al., 2001;Farshori and Kachar, 1999). The α -band migrates at 60 kDa and the β -band migrates at 62 kDa. In this study, 70% of decreased occludin expression in STZ-induced diabetic rats was from the β -band and 30% from the α -band.

Sesamol treatment improved occludin expression only in the β -band. These results indicate sesamol treatment may have restored BBB functional integrity by improving membrane-bound (β -band) but not cytosolic (α -band) occludin expression in STZ-induced diabetic rats.

The several fold reduction in occludin mRNA expression in diabetic rats may be indicative of the diminished ability of the BBB to adapt to insult and infection and decreased occludin and ZO-1 protein expression could be attributed to enzymatic degradation as both are substrates for matrix metalloproteases 2 and 9 (Wachtel et al., 1999; Harkness et al., 2000). Although the mechanisms are unclear, our results indicated that sesamol treatment increased claudin 5 mRNA expression in diabetic rats, which may account for the increased tight junction integrity. While these results are not conclusive, they do suggest that tight junctions are a primary determinant in maintenance and regulation of the paracellular pathway of the BBB and that BBB phenotype may be changed, at both the transcriptional and translational level, during a chronic disease.

We showed for the first time that supplementation with an antioxidant in STZ-induced diabetic rats attenuated BBB permeability. This study indicated that administration of sesamol protects the diabetic brain from oxidative damage by enhancing antioxidant capacity and reducing levels of oxidative stress. The mechanisms by which sesamol enhanced the antioxidant capacity of the brain and prevented BBB breakdown needs to be further elucidated. However, these findings signify that sesamol and/or structurally similar analogues may be therapeutically advantageous in minimizing long-term cerebrovascular complications associated with diabetes.

	Weight (g) gain between 0 and 56 d	Blood glucose (mg/dl) at 56 d	Hb _{A1c} (%) at 56 d
CON	150 ± 5	117 ± 4	4.5 ± 0.1
STZ	41 ± 6 ^a	508 ± 16 ^a	11.7 ± 0.6 ^a
CON+S	131 ± 6	116 ± 4	4.6 ± 0.1
STZ+S	44 ± 5 ^a	501 ± 18 ^a	10.5 ± 0.6 ^a

Data represent mean ± S.E.M. HbA1c, glycosylated hemoglobin. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. ^a denotes a statistically significant (p<0.05) difference as compared to CON.

Table 3.1. Physiological Outcomes Table. Data represent mean \pm S.E.M. HbA1c, glycosylated hemoglobin. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. ^a denotes a statistically significant ($p < 0.05$) difference as compared to CON.

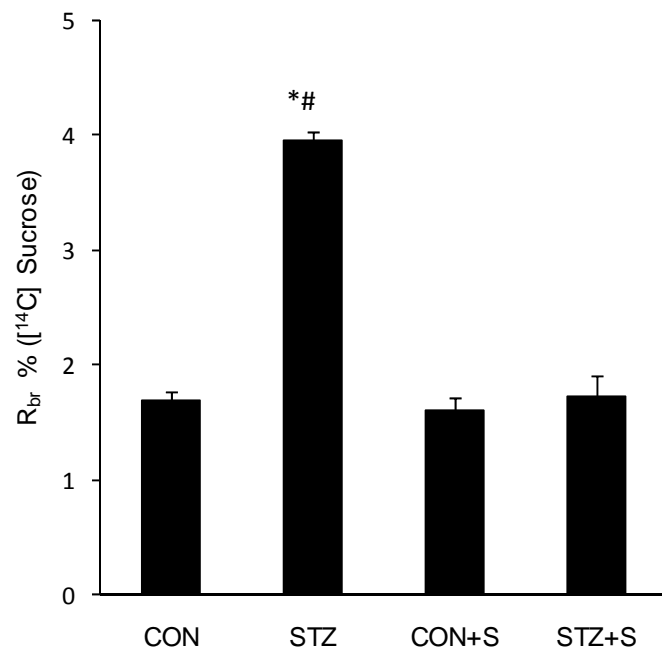


Figure 3.1. *In situ* brain perfusion. Changes in blood-brain barrier permeability during diabetes and the effect of sesamol treatment were measured using a 20 min *in situ* brain perfusion (n=6 rats/group). [¹⁴C]sucrose was used as a vascular space marker and changes in the ratio of sucrose in the brain and perfusate were indicative of altered blood-brain barrier paracellular permeability. Results showed that STZ had significantly increased blood-brain barrier permeability to [¹⁴C]sucrose at 56 d as compared to CON and STZ+S. No difference was observed between CON, CON+S, and STZ+S. Bars represent mean ± S.E.M. * denotes a statistically significant (p<0.05) difference as compared to CON. # denotes a statistically significant (p<0.05) difference as compared to STZ+S.

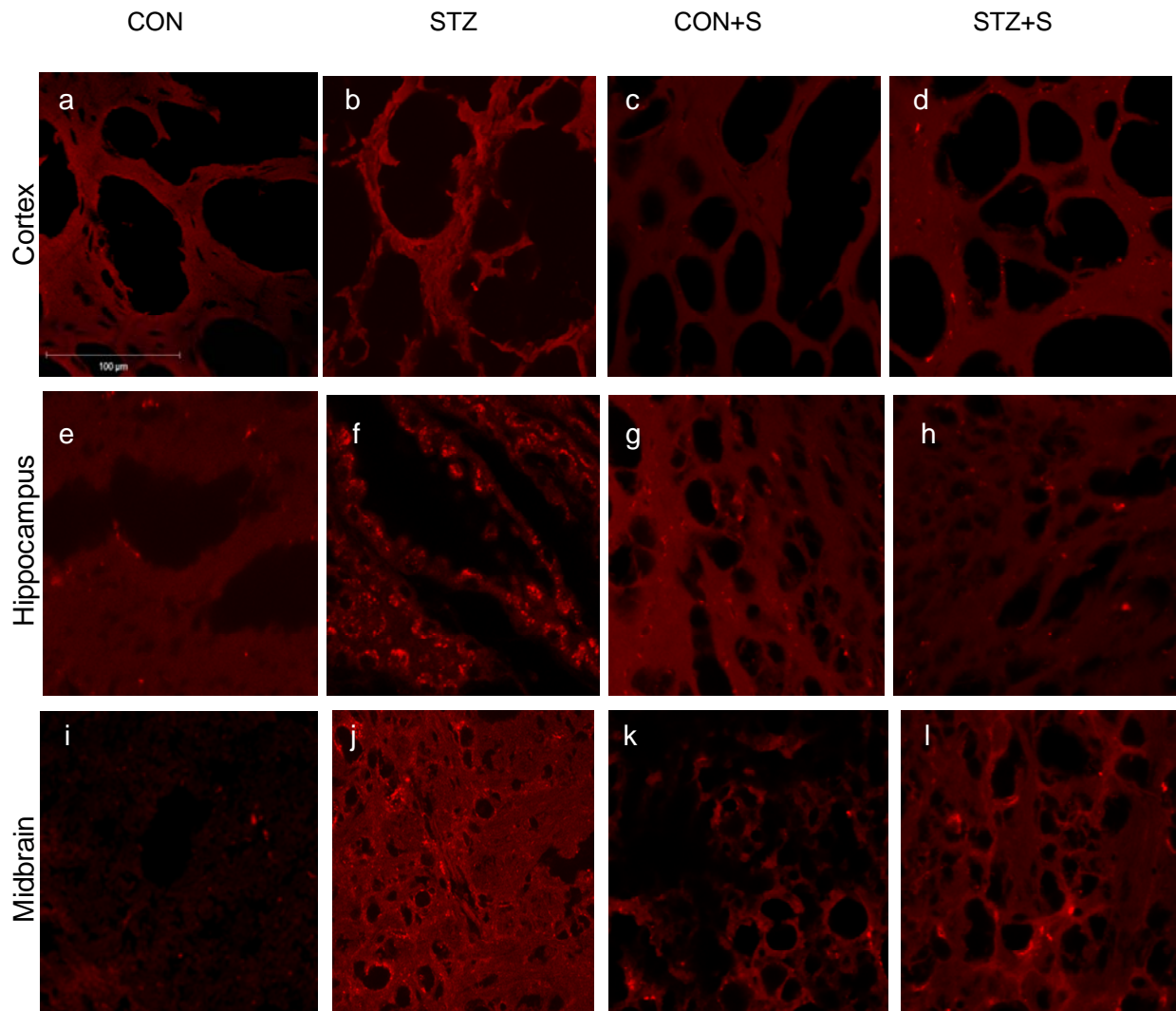


Figure 3.2. Determination of superoxide production. Superoxide production (n=4 rats/group) was assessed by fluorescent detection of the conversion of DHE into ethidine at 56 d. Scale bar =100 μ m. Basal expression of superoxide was shown in cortex (a), hippocampus (e), and midbrain (i) of CON. STZ demonstrated a marked elevation in superoxide production in the cortex (b), hippocampus (f), and midbrain (j). STZ+S showed that sesamol treatment markedly reduced superoxide levels in the cortex (d), hippocampus (h) and midbrain (l) of diabetic animals. No difference in superoxide levels was observed between the treatment groups in the cerebellum, thalamus, and basal ganglia. No difference in superoxide production was observed between CON and CON+S (c, g, k).

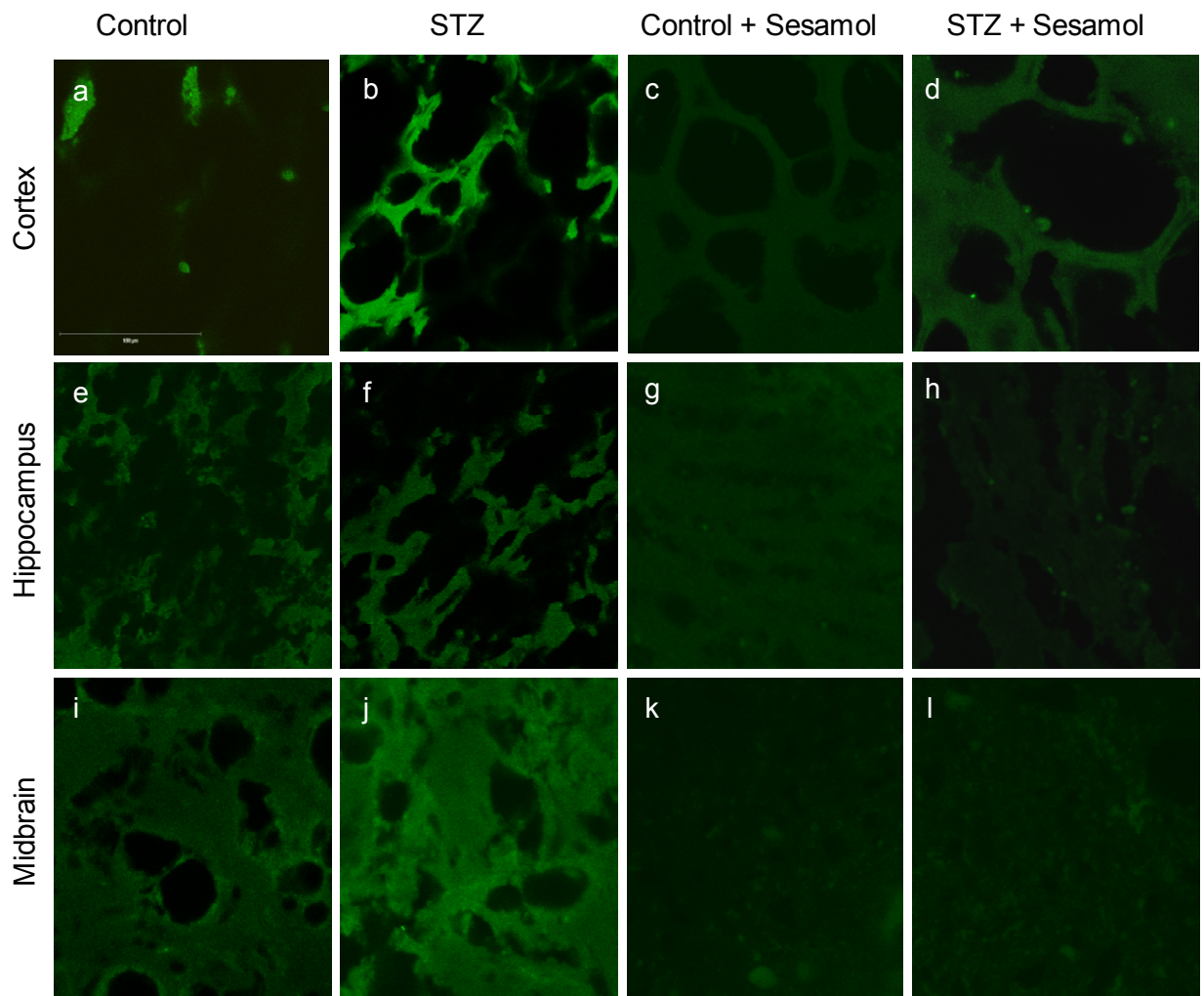


Figure 3.3. Determination of nitrotyrosine production. Nitrotyrosine, a biomarker of peroxynitrite, immunoreactivity (n=4 rats/group) was visualized in brain regions at 56 d using confocal microscopy. Scale bar = 100 μ m. Basal expression of peroxynitrite production was shown in cortex (a), hippocampus (e), and midbrain (i) of CON. STZ showed an elevated peroxynitrite expression in the cortex (b), hippocampus (f), and midbrain (j). STZ+S showed that sesamol treatment reduced peroxynitrite levels in the cortex (d), hippocampus (h), and midbrain (l) but not to the level of CON. No difference in superoxide production was observed between CON and CON+S (c, g, k).

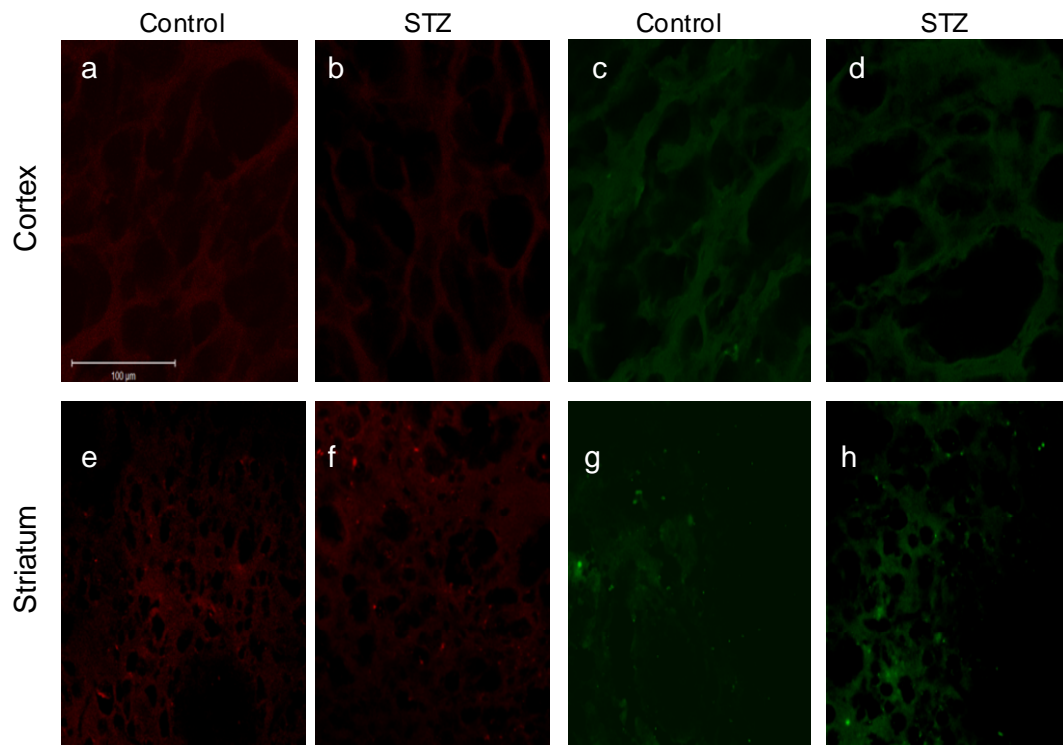


Figure 3.4. Negative control and striatal region showing no change in oxidative stress markers. Representative micrographs of show the negative control of DHE in cortex of CON (a) and STZ (b) and antinitrotyrosine in cortex of CON (c) and STZ (d) rats at 56 d. Scale bar=100 μ m. No difference in DHE was observed between CON (e) and STZ (f) in the striatum. No difference in antinitrotyrosine staining was observed between CON (g) and STZ (h) in the striatum.

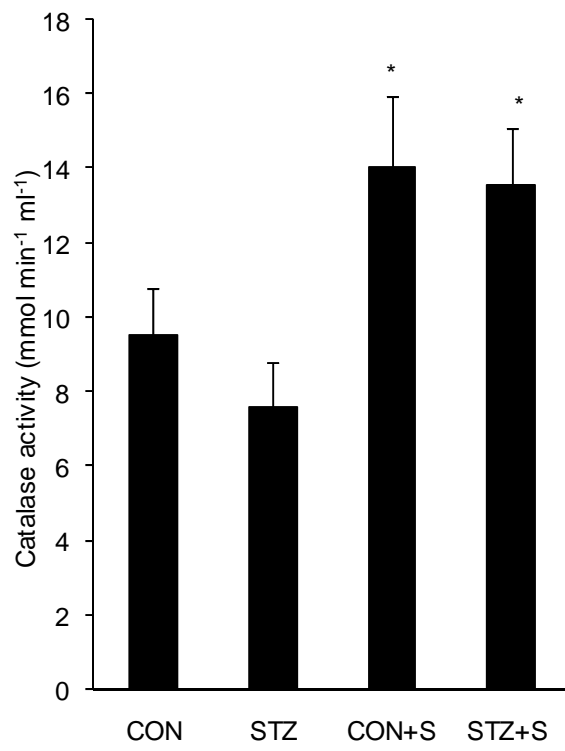
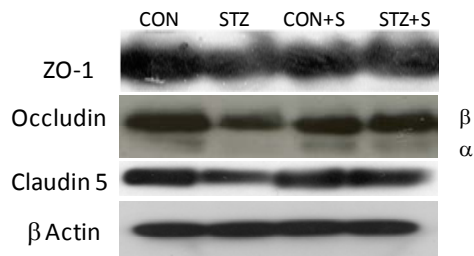
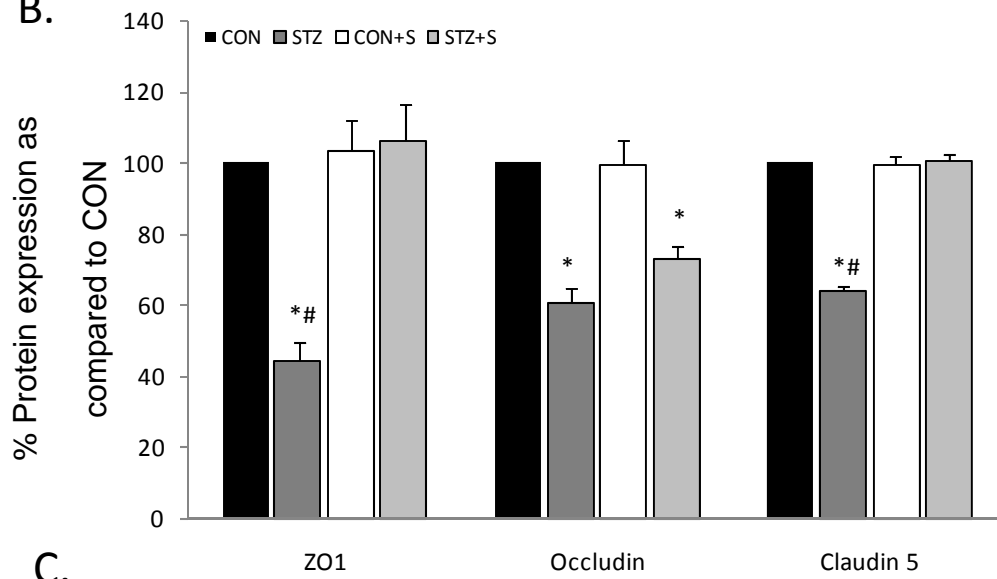


Figure 3.5. Catalase activity. Catalase activity (n=6 rats/group) was measured in brain at 56 d. Basal level (CON) of catalase activity in rats was measured at $9.5 \pm 1.3 \text{ mmol min}^{-1} \text{ ml}^{-1}$. Results showed no effect of diabetes on catalase activity in the brain. CON+S ($13.8 \pm 1.9 \text{ mmol min}^{-1} \text{ ml}^{-1}$) and STZ+S ($14.0 \pm 1.5 \text{ mmol min}^{-1} \text{ ml}^{-1}$) showed increased catalase activity as compared to CON and STZ ($7.6 \pm 1.2 \text{ mmol min}^{-1} \text{ ml}^{-1}$). Bars represent means \pm S.E.M. * denotes a statistically significant ($p < 0.05$) difference as compared to CON.

A.



B.



C.

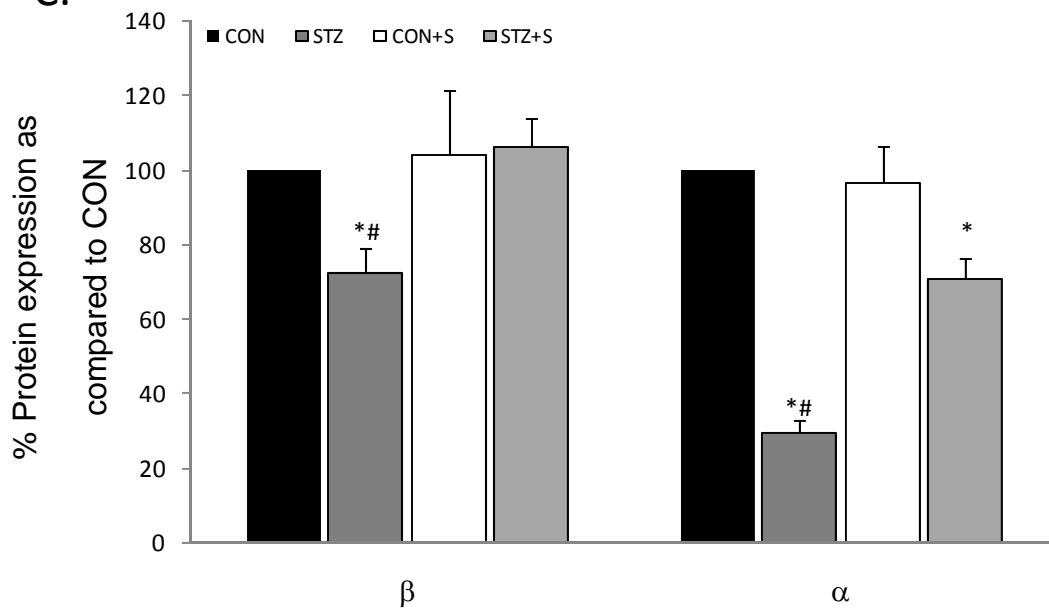


Figure 3.6. Determination of changes in tight junction protein expression. Changes in tight junction protein expression in isolated cerebral microvessels were assessed using western blot and densitometric analysis (n=3 rats/group) at 56 d. **(A)** Representative blot showing expression of denoted proteins in treatment groups. **(B)** STZ showed a significant decrease in claudin 5, occludin, and ZO-1 protein expression as compared to CON and STZ+S. Results indicated a significant effect of sesamol administration between STZ-treated groups for ZO-1 and claudin 5, but not occludin. **(C)** Densitometric analysis showed decreased expression of both α and β occludin bands in STZ rats as compared to CON. A significant decrease in the α band was observed in STZ+S rats as compared to CON and a significant increase in β band expression was observed in STZ+S. Bars represent means \pm S.E.M. * denotes a statistically significant ($p < 0.05$) difference as compared to CON. # denotes a statistically significant ($p < 0.05$) difference as compared to STZ+S.

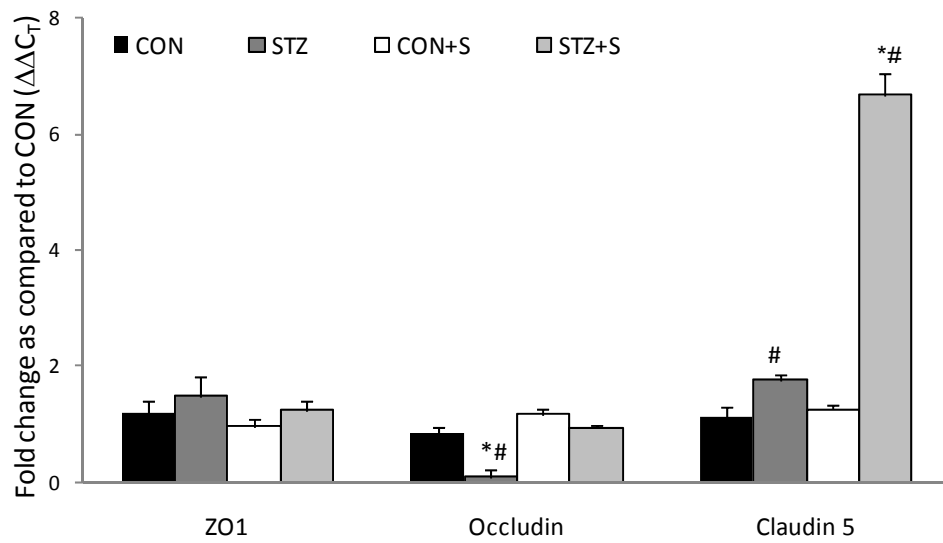


Figure 3.7. Determination of changes in tight junction mRNA expression. Changes in tight junction protein expression in isolated cerebral microvessels were assessed using real time PCR (n=3 rats/group) at 56 d. Results showed no difference in mRNA expression of *zo-1* between the treatment groups as compared to CON. *Occludin* mRNA expression was significantly decreased as compared to CON. Sesamol treatment (STZ+S) increased *occludin* mRNA expression to levels comparable to CON. *Claudin 5* mRNA levels were significantly increased in the STZ and STZ+S groups as compared to CON. Bars represent means \pm S.E.M. * denotes a statistically significant ($p < 0.05$) difference as compared to CON. # denotes a statistically significant ($p < 0.05$) difference as compared to STZ+S.

CHAPTER FOUR

Examining oxidative mechanisms contributing to BBB permeability:

Sesamol reduces lipid peroxidation in the diabetic rat brain

4.1 ABSTRACT

Due to stores of iron and high levels of polyunsaturated fatty acids, the brain is particularly susceptible to Fenton-induced oxidative damage under the pro-oxidant conditions during diabetes. Oxidative stress exacerbates diabetes-induced microangiopathies and consequently abnormal, immature or leaky vessels develop due to insufficient perfusion. These pathophysiological changes have been well-documented in diabetes associated microangiopathies, however, few studies have investigated neovascularization with regards to cerebral microangiopathy. In this study, we administered the antioxidant sesamol and investigated whether Fenton-induced lipid peroxidation was a source of oxidative stress in the diabetic rat brain and whether mechanisms of vascular remodeling or neovascularization were present and/or influenced by oxidative stress. Experiments were conducted at 56 d after STZ injection. Male Sprague-Dawley rats randomly were divided into four treatment groups (CON- control; STZ- STZ-induced diabetes; CON+S- control+sesamol; STZ+S- STZ-induced diabetes+sesamol). Parameters of the Fenton-reaction were assessed by spectrophotometric assay. Signs of vascular remodeling were measured by gel zymography and neovascularization transcripts were quantitated by real time RT-PCR analysis. Results demonstrated that diminished mitochondrial SOD activity and increased lipid peroxidation observed in STZ-induced diabetic was reversed in STZ+S rats. Sesamol treatment reduced PDGF transcription in STZ+S rats, but had no effect on MMP activity. MMP-2 activity was up-regulated in both the STZ and STZ+S groups. In conclusion, this study showed that sesamol treatment enhanced antioxidant capacity and reduced lipid peroxidation in the diabetic brain, but the role of the Fenton-reaction remains unclear. Furthermore, MMP-2 activity does not appear to be modulated by oxidative stress and further investigations are needed to determine if neovascularization occurs in the diabetic brain.

4.2 INTRODUCTION

Oxidative stress exacerbates diabetes-induced microangiopathies by influencing vascular remodeling (Rask-Madsen and King, 2007) and altering hemodynamics (Safar and Lacolley, 2007). A consequence of microangiopathy is the development of abnormal, immature or leaky vessels (Carmeliet, 2005) due to insufficient perfusion. While these pathophysiological changes are well-documented in the retina, kidney and nerve (Figuroa-Romero et al., 2008; Forbes et al., 2008; Kowluru and Chan, 2007), few studies have investigated neovascularization with regards to cerebral microangiopathy associated with diabetes.

Previously, our lab has shown that streptozotocin (STZ)-induced diabetes causes a region specific increase in paracellular permeability at the blood-brain barrier (BBB) (Huber et al., 2006), which coincided with regionally elevated markers of nitrosative and oxidative stress (VanGilder, 2009). Of particular interest, our findings demonstrated that administration of sesamol, a natural antioxidant, attenuated the degree of oxidative stress and maintained the functional and structural integrity of the BBB (VanGilder, et al. 2009). While these results indicate that oxidative stress plays a role in BBB dysfunction, the exact mechanism(s) are not clearly defined.

We propose that the brain, due to high levels of polyunsaturated fatty acids and stores of non-heme iron (Reiter, 1995; Halliwell, 1992), is particularly susceptible to Fenton-induced lipid peroxidation under pro-oxidant conditions associated with long-term uncontrolled or poorly controlled blood glucose. Sesamol is a unique antioxidant that has two functional groups capable of scavenging reactive oxygen species (ROS). Many studies recognize the ROS neutralizing antioxidant properties of a phenolic ring (Kim and Lee, 2004). However, the less studied benzodioxyl moiety scavenges hydroxyl radicals (Kumagai et al., 1991) and slows oxidative chain reactions such as Harber-Weiss and Fenton lipid peroxidation (Okada and Okajima, 1998; Joshi et al., 2005). Following peripheral administration, intact sesamol has been identified in the brain (Jan et al., 2008) and has shown the ability to neutralize Fenton-

induced lipid peroxidation *in vivo* (Chandrasekaran et al., 2008;Hsu et al., 2008) and *in vitro* (Aboul-Enein et al., 2007;Hsu et al., 2007;Joshi et al., 2005).

In this study, we investigated oxidative-stress related mechanisms that may contribute to BBB permeability in the diabetic rat brain. We examined 1) if Fenton-induced lipid peroxidation was a major source of oxidative stress and 2) whether oxidative stress influenced aberrant neovascularization by modulating gelatinase activity and/or altering pro-angiogenic mRNA transcription (Glut-1, PDGF, VEGF). We hypothesized that both processes would be elevated during diabetes and that sesamol treatment would reduce both Fenton-induced lipid peroxidation and neovascularization.

4.3 MATERIALS AND METHODS

Chemicals and Animals. All chemicals used in this study were of molecular biology grade and purchased from Sigma Chemical (St. Louis, MO), unless otherwise noted. Male Sprague-Dawley rats (Harlan; Indianapolis, IN) weighing 250–274 g were housed under 12-h:12-h light-dark conditions and received food and water ad libitum. Rats were acclimated for 7 d before induction of diabetes. Weight and glucose levels were determined at 0 and 56 d of the study. All protocols involving rats were approved by the West Virginia University Animal Care and Use Committee and abide by National Institutes of Health guidelines.

Diabetes induction procedures. STZ was dissolved in sodium citrate (50 mM) buffered saline (vehicle). Sesamol was dissolved in 0.9% saline. All injections were given intraperitoneally (i.p.). Rats were divided into four treatment groups. The first treatment group (CON) received an injection of vehicle and then at 7 d post-injection received an injection of saline (200 μ l) daily until 56 d. The next treatment group (STZ) received an injection of STZ (60 mg/kg; 100 μ l) in vehicle and then at 7 d post-injection received an injection of saline (200 μ l) daily until 56 d. The third treatment group (CON+S) received an injection of vehicle and then at 7 d post-injection received an injection of sesamol (10 mg/kg; 200 μ l) daily until 56 d. The final treatment group (STZ+S) received an injection of STZ (100 μ l) in vehicle and then at 7 d post-injection received an injection of sesamol (200 μ l) daily until 56 d. To avoid early mortality from severe hypoglycemia, rats injected with STZ were supplemented with 10% glucose water for 12 h to prevent mortality from hypoglycemic shock (Huber et al., 2006; Ramachandra et al., 2005; Singh and Jialal, 2008; Cheng et al., 2003; Ramsay and White, 2000; Babu and Srinivasan, 1999). Rats were denoted as having diabetes if blood glucose level measured \geq 350 mg/dl by 2 d post-STZ injection. At the end of the study, animals were anesthetized with sodium pentobarbitol (60 mg/kg) and perfused with ice cold 1 \times PBS (pH 7.4) to remove blood. Due to a high microvessel density, cortical tissue was used for this study.

Hydrogen peroxide production. H₂O₂ production was assessed using the spectrophotometric assay Amplex Red hydrogen peroxide/peroxidase assay (Molecular Probes, Eugene, OR). Cortex tissue (100 mg) was homogenized in 1:10 (w/v) in 1× PBS (pH 7.4) with protease inhibitors and spun down at 10,000 g. Supernatant was stored at -80° C until assay was performed. Reaction mixtures contained 50 µM Amplex Red reagent, 0.1 units/ml peroxidase. Hydrogen peroxide standards were treated the same as samples. Standards and samples were assayed in duplicate and read at 540 nm on a SpectroMax 340PC.

Iron quantification. Ferrous iron levels were measured spectrophotometrically using the reaction between Ferrozine and Fe²⁺ (Smith et al., 1998; White and Flashka, 1973). Cortex tissue (~50 mg) was homogenized 1:10 (w/v) in acetate buffer (0.15M sodium acetate, pH 4.5). Ferrozine (38mM;50:1) dissolved in acetate buffer and 50 µl of 10% TCA was added to the homogenate and vortexed briefly. FeCl₂ standards (dissolved in acetate buffer) were treated the same as samples. Standards and samples were incubated for 20 min at 37° C and centrifuged for 30 min at 15,000 g at 4° C. Standards and samples were assayed in duplicate and read at 560 nm on a SpectroMax 340PC.

Thiobarbituric Acid Reactive Substances (TBARS) estimation. Lipid peroxidation was measured spectrophotometrically using the reaction between malondialdehyde (MDA) with thiobarbituric acid (TBA) (Ohkawa et al., 1979). Cortex tissue (~25 mg) was homogenized 1:10 (w/v) in RIPA buffer with protease inhibitors. After a brief spin to remove brain debris, supernatant was stored at -80 ° C until assay was performed. Addition of 100 µl 14% SDS and 4 mls of 0.8% TBA in 20% acetic acid, pH 4.0 to 100 µl of the supernatant. MDA standards were treated the same as samples. Standards and samples were boiled for 1 h at 95°c, incubated on ice for 10 min and centrifuged 1,600 g at 4° C for 10 min. Standards and samples were assayed in duplicate and read at 540 nm on a SpectroMax 340PC.

Superoxide Dismutase activity. Cytosolic and mitochondrial SOD activities were measured using a commercial kit from Cayman Chemicals. Cortex tissue was homogenized (1:10, w/v) in cold 20 mM

HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mM mannitol and 70 mM sucrose. Samples were spun at 1,500 g for 5 min at 4° C to remove tissue debris. Cytosolic and mitochondrial SOD were separated by centrifugation at 10,000 g for 15 min at 4° C. Supernatant was removed and mitochondrial pellet was resuspended in HEPES buffer with 1 mM KCN. Both fractions were stored at -80 ° C and assayed according to the manufacturer's instructions. Standards and samples were assayed in duplicate and read at 450 nm on a SpectroMax 340PC.

Gel zymography. Serum was collected without an anticoagulant, allowed to clot for 30 min at room temperature and then spun at 2,000 g for 15 min at 4°C. Serum was saved and stored at -80°C until use. Plasma samples (1 µl) were run with molecular mass markers and recombinant MMP-2 and MMP-9 standards (Sigma) under non-reducing conditions (without β -mercaptoethanol or dithiothreitol) on 10% Tris-HCl gels containing 0.5% gelatin (Novex; Invitrogen). Enzymes were renatured in the gel with 2.5% Triton X-100 in deionized water for 1h at room temperature. Gels were then equilibrated in a digestion buffer (5 mmol/l CaCl₂, 50 mmol/l Tris-HCl, pH 7.4, 200 mmol/l NaCl, and 0.2% Brij35) for 45 min at room temperature and then incubated for 12h at 37°C. Gels were stained with .5% Brilliant Blue G (Biorad) for 1 h, followed by destaining in multiple washes with 5% methanol–7.5% acetic acid (until wash solution was clear, approximately 2 h, then photographed and analyzed with a FlourChem SP (AlphaInnotec, Inc.). Clear bands were indicative of gelatinase activity.

Determination of changes in vascular remodeling mRNA expression in the cortex. Total RNA was isolated from cortex using TriReagent[®]. Concentration and purity of RNA was determined using a biophotometer and considered for use only if A₂₆₀/A₂₈₀ was ≥ 1.80 . Total RNA was reverse transcribed into cDNA using a 20 µl reaction. Real time PCR analyses of *MMP-2*, *MMP-9*, *Glut-1*, *PDGF* and *VEGF* using a StepOne™ detection system (Applied Biosystems; Foster City, CA) in combination with TaqMan[®] chemistry. Specific primers and dual labeled internal (FAM/TAMRA) probe sets were used according to manufacturer's recommendation (Applied Biosystems). All PCR amplifications were run in a 20 µl reaction volume consisting of 1µl cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

was used as an endogenous control to normalize for differences in amount of cDNA added to reactions. Negative controls were monitored simultaneously within each run. Thermocycling conditions were set to holding stage at 50° C for 2 min and 95°C for 10 min and 40 cycles of 95° C for 15 s and 60° C for 1 min. According to manufacturer's instructions, relative quantification of mRNA transcripts was carried out using the comparative threshold (ΔC_T) method.

Statistical analysis. Data were expressed as mean \pm S.E.M. Statistical analyses were performed using two way analysis of variance (ANOVA) followed by Tukey's post hoc test. Level of significance was set at $p < 0.05$.

4.4 RESULTS

Determination of hydrogen peroxide production. Hydrogen peroxide levels (n=6 rats/group) were measured in the cortex at 56 d (Figure 4.2 A). A significant interaction between disease state and sesamol treatment [$F_{1,23}=7.8;P=0.01$] was observed. Results indicated elevated hydrogen peroxide levels in STZ ($3.9\pm 0.2 \mu\text{M H}_2\text{O}_2$) as compared to CON ($2.4\pm 0.2 \mu\text{M H}_2\text{O}_2$). Sesamol treatment significantly altered hydrogen peroxide levels between CON ($2.4\pm 0.2 \mu\text{M H}_2\text{O}_2$) and CON+S ($2.9\pm 0.1 \mu\text{M H}_2\text{O}_2$), but no difference was noted between STZ ($3.9\pm 0.2 \mu\text{M H}_2\text{O}_2$) and STZ+S ($3.3\pm 0.2 \mu\text{M H}_2\text{O}_2$).

Determination of Fe^{2+} levels. Fe^{2+} quantification (n=6 rats/group) was performed on cortical tissue at 56 d (Figure 4.2 B). Results indicated no difference in Fe^{2+} levels between disease state [$F_{2,24}=0.22;P=0.81$] or sesamol treatment [$F_{1,24}=0.08;P=0.79$]. Basal Fe^{2+} levels were noted in CON ($240\pm 8 \mu\text{g Fe}^{2+} / \text{mg}$ tissue).

TBARS Estimation. TBARS levels (n=6 rats/group) were determined in cortical tissue at 56 d (Figure 4.2 C). Both disease [$F_{1,24}=7.4;P=0.013$] and sesamol treatment [$F_{1,24}=15.0;P<0.001$]. revealed significant interactions. STZ ($354\pm 12 \text{ nM MDA} / \text{mL}$) showed a significant increase in lipid peroxidation as compared to CON ($301\pm 18 \text{ nM MDA} / \text{mL}$). CON+S ($254\pm 10 \text{ nM MDA} / \text{mL}$) and STZ+S ($285\pm 14 \text{ nM MDA} / \text{mL}$) showed decreased lipid peroxidation levels when compared to CON and STZ, respectively.

Superoxide Dismutase Activity. Cytosolic and mitochondrial SOD levels (n=6 rats/group) were analyzed in the cortex at 56 d (Figure 4.3). (A) Results showed significantly reduced mitochondrial SOD activity in STZ ($52\pm 2 \text{ U} / \text{mL}$) as compared to CON ($119\pm 2 \text{ U} / \text{mL}$) [$F_{1,17}=23.6;P<0.001$]. Sesamol treatment enhanced mitochondrial SOD activity when compared to saline treatment [$F_{1,17}=8.2;P=0.01$]. Elevated mitochondrial SOD activity was observed in CON+S ($134\pm 10 \text{ U} / \text{mL}$) and STZ+S ($81\pm 12 \text{ U} / \text{mL}$) when compared to the respective saline treated-disease state. (B) Diabetes [$F_{1,20}=0.06;P=0.81$] or sesamol treatment [$F_{1,20}=0.59;P=0.45$] did not show differences among cytosolic SOD activities.

Determination of gelatinase activity in serum. MMP-2 and MMP-9 activity (n=3 rats/group) were analyzed in rat serum at 56 d using gel zymography (Figure 4.4). Pro-MMP-2 showed a activity differences between CON and STZ [$F_{1,11}=17.4;P=0.003$], but not between saline and sesamol treatment [$F_{1,11}=2.4;P=0.16$]. Likewise, active-MMP revealed a difference between CON and STZ [$F_{1,11}=5.81;P=0.04$], but not between saline and sesamol treatment [$F_{1,11}=1.16;P=0.24$]. No difference in MMP-9 activity was observed for diabetes [$F_{1,11}=1.11;P=0.32$] or significantly reduced with sesamol treatment [$F_{1,11}=0.93;P=0.36$].

Determination of changes in vascular remodeling mRNA expression in the cortex. Real time PCR (n=3 rats/group) was performed to measure changes in gene expression of gelatinases (*MMP-2* and *MMP-9*) and neovascularizing transcripts (*VEGF*, *GLUT-1*, and *PDGF*) at 56 d (Table 1). Changes in gene expression were calculated based on the ΔC_T method with GAPDH serving as the endogenous control. No difference in *MMP-2*, *MMP-9* or *VEGF* mRNA expression was observed between any groups. *GLUT-1* showed increased mRNA expression in STZ (56 fold), CON+S (51 fold), and STZ+S (56 fold) as compared to CON. *PDGF* gene expression was elevated in STZ and STZ+S as compared to CON (11 fold and 2 fold, respectively).

4.5 DISCUSSION

The major findings of the study were that sesamol treatment reduced cortical lipid peroxidation and enhanced mitochondrial SOD activity in STZ induced diabetic rats. Contrary to our hypothesis, results showed no difference in ferrous iron concentration and we were not able to establish whether iron plays a role in cortical lipid peroxidation during STZ-induced diabetes. Oxidative stress appears to affect neovascularization as we observed elevated PDGF transcription in the STZ group and reduced PDGF transcription in the STZ+S group. Meanwhile, vascular remodeling as indicated by MMP-2 activity is enhanced during diabetes and is not affected by sesamol treatment.

Iron oxidation and accumulation have been implicated in many neurological and neurodegenerative diseases and may be a consequence of regionally altered vascular changes (Brun and Englund, 1986; Snowdon, 2003; Faucheux et al., 1999). Our results did not reflect differences in ferrous iron concentration; however, this study did not encompass changes in iron storage. Altered iron metabolism occurs with age (Hirose et al., 2003; Connor et al., 1990) and may be relevant to accelerated aging seen in the diabetic brain. In the brain, iron is stored in ferritin complexes. Altered ferritin isoform expression is indicative of cell stress responses and this change has been documented in aged human cortex (Connor et al., 1992). Furthermore, oxidatively damaged ferritin, hemosiderin, may not adequately store iron, thus making it more reactive. (Zecca et al., 2004). Additional studies regarding iron metabolism during diabetes are needed.

While we were unable to conclusively determine that iron dysregulation played a role in elevated lipid peroxidation in STZ-induced diabetic rats, we did observe decreased lipid peroxidation in both CON and STZ sesamol-treated groups. It is plausible that decreased lipid peroxidation seen with sesamol is due to gene regulating abilities and antioxidant chemical properties that enable sesamol to neutralize hydroxyl and peroxy radicals (Uchida et al., 1996; Hsu et al., 2007). Studies show that sesame seed lignans are able to decrease transcription of lipogenic enzymes and enhance transcription of alcohol-metabolizing

enzymes (Tsuruoka et al., 2005;Kiso et al., 2005;Arachchige et al., 2006). Specifically, sesamol is generated from the roasting of sesamol, a compound that decreases hepatic fatty acid synthesis and dose-dependently enhances hepatic fatty acid oxidation (Ide et al., 2009;Lim et al., 2007). Similar mechanisms may account for decreased lipid peroxides and enhanced catalase activity in the brain observed in our previous study (VanGilder et al., 2009).

Excessive hydrogen peroxide forms hydroxyl radical, which initiates lipid peroxidation and impairs enzyme activity by oxidizing metal cofactors (Reiter, 1995;Halliwell, 1992). In this study, we measured hydrogen peroxide production as an indirect measure of hydroxyl radical formation. Furthermore, we assayed cytosolic and mitochondrial SOD activity because both enzymes contain essential metal co-factors that are susceptible to hydroxyl radical damage. We observed elevated hydrogen peroxide levels and decreased mitochondrial SOD activity in STZ-induced diabetic rats. Hydrogen peroxide producing enzymes, xanthine oxidase, NADPH oxidases, or glucose oxidase, are up-regulated during diabetes (Thomas et al., 2008;Munzel et al., 2008). In turn, hydroxyl radical formation could explain the decreased mitochondrial SOD activity. Additionally, sesamol treatment increased mitochondrial SOD activity, which may account for the elevated hydrogen peroxide levels seen in CON+S. Although we did not observe changes in hydrogen peroxide levels between STZ and STZ+S groups, we have previously shown reduced oxidative stress markers in the cortex (VanGilder et al., 2009). Radical-inhibiting and gene regulating properties of sesamol may better equip the diabetic brain to handle oxidative stress.

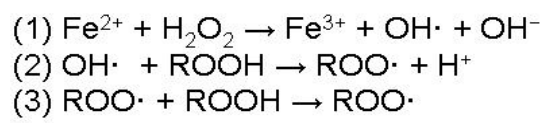
Oxidative stress during diabetes can influence transcription and activity of MMPs (Shin et al., 2008;Galli et al., 2005;Haorah et al., 2007), which are known for their physiological role in vascular remodeling. Our results showed no changes in transcription and previous reports demonstrated no changes in MMP-2 or MMP-9 activity in the diabetic rat brain (Hawkins et al., 2007). However, signs of oxidative stress that exist in the systemic circulation can effect microvascular function (Martin-Gallan et al., 2007). Other studies have shown enhanced gelatinase in the serum of patients (Derosa et al., 2004)

and rats with type 1 diabetes (Hawkins et al., 2007). Consequently, elevated circulating MMPs could contribute to BBB permeability by degrading basement membrane proteins (collagen IV, laminin, etc) and tight junction proteins (Harkness et al., 2000;Wachtel et al., 1999). In our study, STZ-induced diabetes showed elevated MMP-2 activity with no differences between pro and active MMP-2 activity in the STZ and STZ+S groups. MMPs are released as an inactive pro-enzyme that is catalytically activated by another protease, however, oxidation or nitration of the inhibitory cysteine residue can prematurely activate the pro-zymogen (Gu et al., 2005). Sesamol treatment does not appear to modify MMP-2 expression or activity during diabetes. These results indicate that circulating MMP-2 is not modulated by sesamol and may be upregulated due to pro-inflammatory cytokines seen during diabetes (Adya et al., 2008;Chow et al., 2007;Gurjar et al., 2001). Furthermore, we observed no changes in circulating MMP-9 activity, which is not surprising because elevated MMP-9 activity is often related to inflammation during acute injury (Park et al., 2009;Hayashi et al., 2009). These results suggest that oxidative stress is not the primary mechanism promoting systemically circulating gelatinases during diabetes and that another mechanism such as inflammation may be the underlying cause.

Vascular remodeling and aberrant neovascularization result from pro-oxidant induced microvascular dysfunction observed in the retina. The blood-retinal barrier is analogous to the BBB in that it selectively regulates the local environment (i.e. osmotic balance, ionic concentration, and transport of nutrients) of the neural retina (Giebel et al., 2005). An imbalance between supply and demand for oxygen and nutrients leads to pathological angiogenesis (Fraisl et al., 2009). To discern if similar mechanisms occurred in the diabetic brain, we examined neovascularizing transcripts in the cortex. The GLUT-1 transporter is responsible for glucose transport in endothelial cells of the retina and brain and can also be found on brain astrocytes (Abbott, 2002). While hyperglycemia decreases GLUT-1 transcription and expression in retina endothelial cells (Jacob et al., 2002;Taarnhoj and Alm, 1991), studies on brain endothelial cells are conflicted showing either decreased or no changes in GLUT-1 transcription or expression (Mayhan, 1997;McCall et al., 1984;Knudsen et al., 1986). However, our study

showed a 55-fold increase in GLUT-1 expression in STZ, CON+S, and STZ+S groups. Metabolic changes in astrocytes due to hyperglycemia or sesamol treatment may explain these differences in transcription. Because astrocytes can secrete factors that modulate BBB function, further investigation is warranted. PDGF and VEGF can mediate tight junction redistribution and increased vessel permeability (Harhaj et al., 2006;Antonetti et al., 1998;Harhaj et al., 2002). In our study, PDGF showed an 11-fold increase in our STZ-induced diabetic group, which was reduced to approximately 2-fold in STZ+S. Our results did not show a difference in VEGF transcription, however, examining cortical microvessels instead of whole brain tissue may condense transcripts and yield transcription differences. The difference observed in PDGF transcription may be indicative of dysregulation of the neurovascular unit, in which case further investigations defining the roles of pro-inflammatory cytokines and neovascularizing factors are needed.

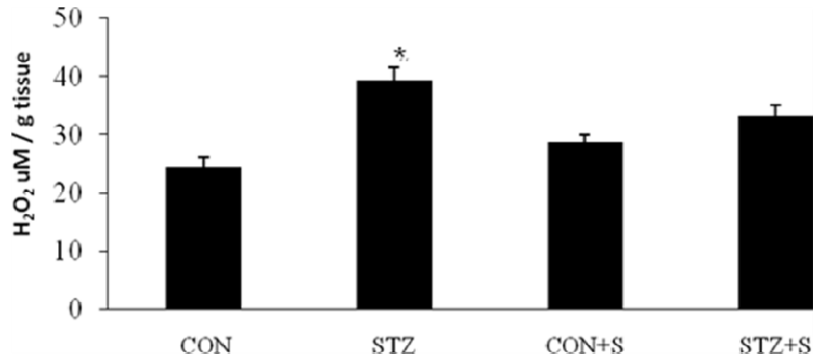
We showed that sesamol treatment reduced PDGF transcription in STZ-induced diabetic rats; thus indicating that signs of neovascularization are present and can be modulated with antioxidant therapy. Enhanced mitochondrial SOD activity and decreased lipid peroxidation with sesamol treatment suggest mitochondrial lipogenesis may be affected in the brain. Furthermore, sesame seed lignans are able to reduce lipid peroxidation in the liver by modulating lipolytic gene expression. It is likely that sesamol follows similar mechanisms in the brain. Sesamol and sesame lignans may be beneficial in treating lipid peroxidation organ damage and dyslipidemia observed in both type 1 and type 2 diabetes.



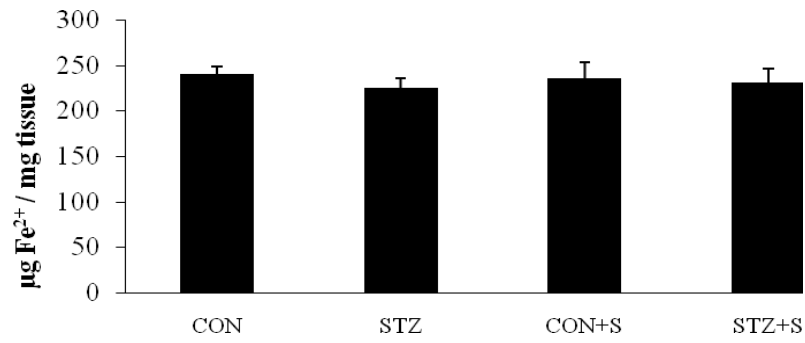
Fenton Reaction
Hydroxyl Initiated Lipid Peroxidation
Self-propagating Lipid Peroxidation

Figure 4.1 Schematic of Fenton Induced Lipid Peroxidation. (1) Labile iron in the brain can be oxidized by elevated hydrogen peroxide. This reaction forms hydroxyl anion. (2) Hydroxyl anion initiates lipid peroxidation. (3) Lipid peroxidation is a self-propagating reaction that can be particularly damaging to the brain due to its high levels of polyunsaturated fatty acids.

A)



B)



C)

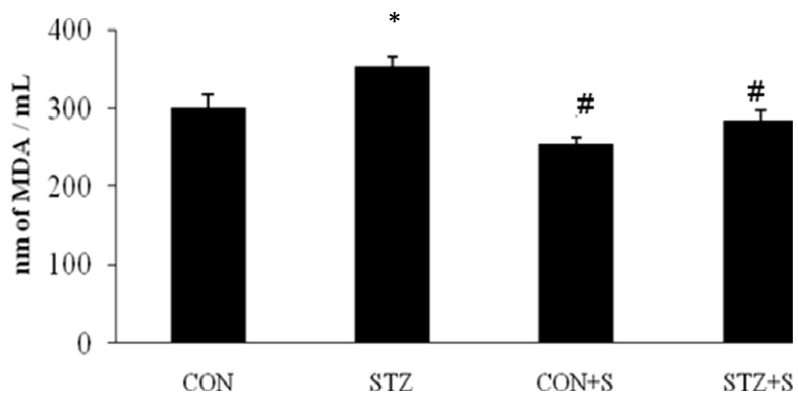
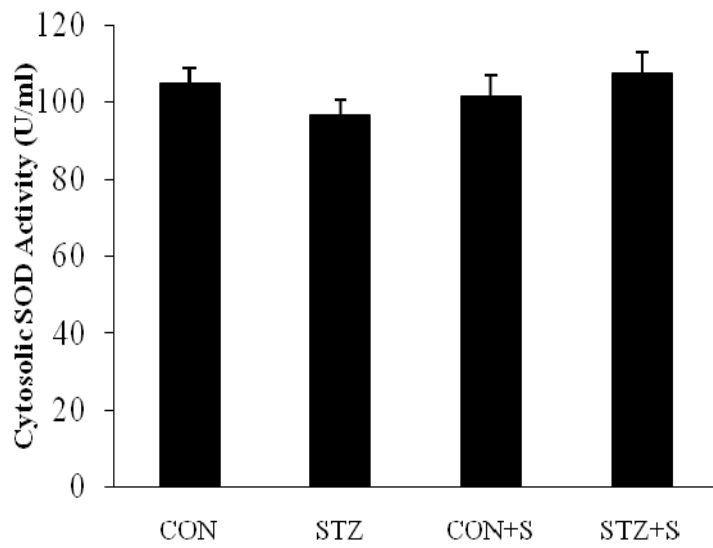


Figure 4.2 Examination of Fenton-Induced Lipid Peroxidation in the Brain. Estimation.

Lipid peroxidation (n=6 rats/group) was measured in the cortex at 56d. Basal levels (CON) of lipid peroxidation measured at 300.5 ± 18.0 nm MDA ml^{-1} . STZ (354.2 ± 11.7 nM MDA /mL) showed elevated lipid peroxidation as compared to CON. CON+S (253.6 ± 10.0 nM MDA / mL) and STZ+S (284.5 ± 13.8 nM MDA / mL) showed decreased lipid peroxidation levels when compared to CON and STZ. **(B) Determination of Fe^{2+} levels.** Fe^{2+} quantification (n=6 rats/group) was performed on cortical tissue at 56 d. Results indicated no difference in Fe^{2+} levels between diabetes or sesamol treatment. **(C) Hydrogen peroxide production.** Hydrogen peroxide levels (n=6 rats/group) were measured in the cortex at 56 d. Basal levels (CON) of hydrogen peroxide were measured to be 2.4 ± 0.2 $\mu\text{M H}_2\text{O}_2$. STZ (3.9 ± 0.2 $\mu\text{M H}_2\text{O}_2$) and CON+S (2.9 ± 0.1 $\mu\text{M H}_2\text{O}_2$) showed elevated hydrogen peroxide production as compared to CON. Bars represent mean \pm S.E.M. * denotes a statistically significant ($p < 0.05$) difference as compared to CON. # denotes a statistically significant ($p < 0.05$) difference as compared to STZ+S.

A)



B)

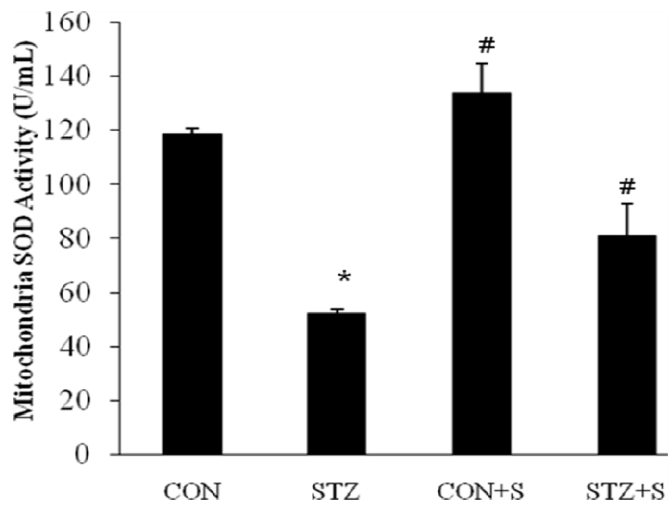
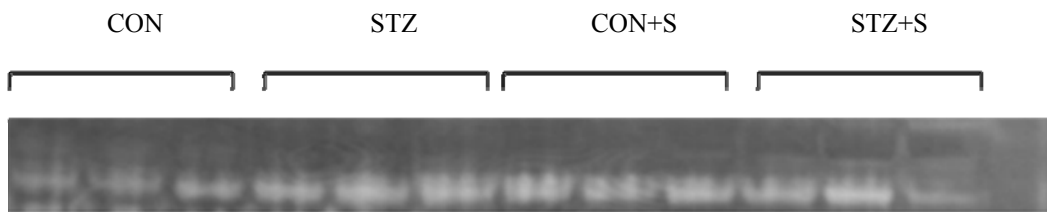


Figure 4.3 Superoxide Dismutase activity. Cytosolic and mitochondrial SOD activity (n=6 rats/group) were analyzed in the cortex at 56 d. (A) Basal activity (CON) of cytosolic SOD was measured at 104.7 ± 4.1 U / mL . Results indicated no difference in cytosolic SOD activity between diabetes or sesamol treatment. (B) Basal activity (CON) of mitochondrial SOD was measured to be 118.6 ± 1.9 U / mL. Elevated activity was observed in CON+S (133.9 ± 10.4 U / mL) and STZ+S (81.2 ± 11.7 U / mL) when compared CON and STZ. Bars represent mean \pm S.E.M. * denotes a statistically significant ($p < 0.05$) difference as compared to CON. # denotes a statistically significant ($p < 0.05$) difference as compared to STZ+S.

A) MMP-2



B) MMP-9



C)

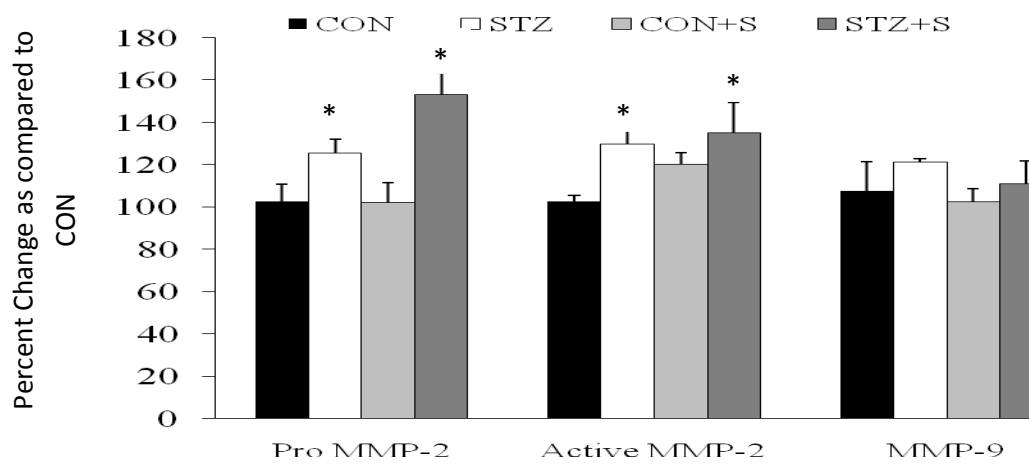


Figure 4.4 MMP-2 and MMP-9 activity in serum. MMP-2 and MMP-9 activities (n=3 rats/group) were analyzed in rat serum at 56 d using gel zymography and densitometry. Pro-MMP-2 and active-MMP-2 showed enhanced activity during STZ-induced diabetes. Sesamol treatment did not affect MMP-2 activity. No differences in MMP-9 activity were observed for diabetes or sesamol treatment. * denotes a statistically significant ($p < 0.05$) difference as compared to CON.

Table 4.1. Vascular Remodeling and Neovascularizing mRNA transcripts (ΔC_t)

mRNA transcripts (ΔC_t)	CON	STZ	CON+S	STZ+S
MMP-2	11.1 \pm 1.0	12.5 \pm 0.7	12.3 \pm 0.6	12.7 \pm 0.3
MMP-9	13.1 \pm 0.5	14.4 \pm 0.4	13.6 \pm 0.1	13.5 \pm 0.3
Glut-1	12.9 \pm 0.4	7.1 \pm 0.8 *	7.2 \pm 0.5 *	7.1 \pm 0.3 *
VEGF	8.2 \pm 0.4	7.8 \pm 0.3	7.9 \pm 0.1	7.7 \pm 0.2
PDGF	6.3 \pm 0.5	2.9 \pm 0.1 *	5.2 \pm 0.3 *	5.4 \pm 0.2 *

Rats were sacrificed at 56d and RNA was isolated from cortical tissue. Changes in mRNA were determined by real-time PCR and the values expressed as ΔC_t as compared to CON. Values were standardized internally to GAPDH expression. Data represent mean \pm S.E.M. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. * denotes a statistically significant ($p < 0.05$) difference as compared to CON.

CONCLUSION

The present studies demonstrate a relationship between diabetes induced-oxidative stress and BBB dysfunction. Our assessment of regional BBB dysfunction and oxidative stress markers correlates to clinical complications seen in patients with diabetes. Patients with diabetes have increased risks for cognitive dysfunction and lacunar infarcts, which are analogous to regions with increased permeability--the cortex and hippocampus or midbrain, respectively. We have shown that the natural antioxidant sesamol is able to reduce BBB permeability and lipid peroxidation in our STZ-induced diabetes rats. Our data and other findings suggest that sesamol and sesamol-related compounds (paroxetine or other sesame lignans) could have beneficial effects as adjuvant therapies for treating microangiopathies and/or dyslipidemia during diabetes. Further studies are needed to define the antioxidant mechanism of sesamol in the brain. Likewise, the BBB and BRB share commonalities in their structure and function, which indicate pathological changes could be similar. While on-going research tries to elucidate mechanisms for retinopathies, clinical diagnostics are able to monitor disease progression. Currently, cerebral microangiopathies are not recognized as a diabetes-associated complication, however, clinical tools such as MRI would allow clinicians to assess cerebral microvascular health. Such measures would give incite to the progression and complications associated with cerebrovascular disease.

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VanGilder RL, Kelly KA, Chua MD, Ptachinkinski RL, Huber JD. Administration of sesamol improved blood-brain barrier function in streptozotocin-induced diabetic rats. Accepted Exp Br Res. 2009.

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Abstracts:

VanGilder RL, Houser KA, Huber JD: Streptozotocin-induced diabetes progressively increases BBB permeability in specific brain regions in rats. Van Liere Convocation, Morgantown, WV. Poster presentation. March 2006

VanGilder RL, Kelly KA, Clyburn KD, Huber JD: STZ-induced diabetes regionally elevates markers of oxidative stress in the brain. Society of Neuroscience, San Diego, CA. Poster presentation. Nov. 2007

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VanGilder RL, Houser KA, Chua MD, Ptachinkinski RL Huber JD: Sesamol restores blood-brain barrier function and structure during STZ-induced diabetes. Van Liere Convocation, Morgantown, WV. Poster presentation. April 2008

Presentations:

Brain Uptake of intranasally applied radioiodinated leptin in Wistar Rats. Fall 2006.

NOS Isoforms Undertake Unique Roles During Excitotoxicity. Spring 2007.

The Effect of Experimental Type 1 Diabetes on Blood Brain Barrier Function and Structure. Fall 2007.

Sesamol Reduces Oxidative Stress and Enhances Antioxidant Capacity in the Diabetic Rat Brain. Spring 2008.

Sesamol Improves BBB structure and Function in STZ-induced Diabetic Rats. Fall 2008.

Sesamol Reduces Lipid Peroxidation in the Diabetic Rat Brain. Spring 2009