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Traumatic Brain Injury Causes Endothelial Dysfunction In Mesenteric Arteries 24 Hrs After Injury

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TRAUMATIC BRAIN INJURY CAUSES ENDOTHELIAL DYSFUNCTION IN MESENTERIC ARTERIES 24 HRS AFTER INJURY

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ABSTRACT

Traumatic brain injury (TBI) is the most frequent cause of death in children and young adults in the United States. Besides emergency neurosurgical procedures, there are few medical treatment options to improve recovery in people who have experienced a TBI. Management of patients who survive TBI is complicated by both central nervous system and peripheral systemic effects. The pathophysiology of systemic inflammation and coagulopathy following TBI has been attributed to trauma-induced endothelial cell dysfunction; however, there is little knowledge of the mechanisms by which trauma might impact the functions of the vascular endothelium at sites remote from the injury. The endothelium lining these small vessels normally produces nitric oxide (NO), arachidonic acid metabolites, and endothelial-dependent hyperpolarizing factors to relax the surrounding vascular smooth muscle. For this research study we investigated the effects of fluid-percussion-induced TBI on endothelial-dependent vasodilatory functions in a remote tissue bed (the mesenteric circulation) 24 hours after injury. We hypothesized that TBI causes changes in the mesenteric artery endothelium that result in a loss of endothelial-dependent vasodilation. We found that vasodilations induced by the muscarinic-receptor agonist, acetylcholine, are attenuated following TBI. While the endothelial-derived hyperpolarizing component of vasodilation was preserved, the NO component was severely impaired. Therefore, we tested whether the loss of NO component was due to a decrease in bioavailablity of the NO synthase (NOS) cofactor BH_4, the NOS substrate L-arginine, or to changes in expression/activity of the enzyme arginase, which competes with NOS for L-arginine. We found that supplementation of L-arginine and inhibition of the enzyme arginase rescues endothelial-dependent vasodilations in TBI arteries. This study demonstrates that there are pathological systemic effects outside the point of injury following TBI leading to a dysfunctional endothelial vasodilatory pathway. These data provide insight into the pathophysiology of endothelial dysfunction after trauma and may lead to new potential targets for drug therapy.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. Epidemiology

Traumatic brain injury (TBI) represents the leading cause of morbidity and mortality worldwide in individuals under the age of 45, and is increasingly recognized as a major health care problem in the United States [73]. According to the US Department of Health and Human Services, there are approximately 1.7 million TBIs each year, resulting in 52,000 deaths, 275,000 hospitalizations and 1,365,000 emergency department visits [33]. The reported data may underestimate the true amount of TBI-related injuries due to possible misdiagnosis, unreported outpatient treatment, and patients who do not seek health care after injury. Despite increased helmet use and safety measures, recent data indicate increasing emergency department visits for TBI in the US; this may be due in part to increased awareness, exposure, and diagnostics [76]. People who survive brain injury can suffer from long-term effects diminishing their productivity and overall quality of life. It has been estimated that more than 124,000 additional people each year will suffer from significant long-term disabilities after severe brain trauma [117]. Some individuals develop dementia and chronic traumatic encephalopathy from more mild, often repetitive, concussions [117]. Long-term effects can also arise from TBI and can include pathological manifestations such as seizures, epilepsy, neurodegeneration and Alzheimer's disease [32]. Thus, the total cost to society and to our health care system from TBI is enormous.

The most common mechanism of injury resulting in a TBI is through a fall, but this is most prevalent in older adults [33]. Motor vehicle accidents, almost as common, account

for the largest proportion of TBI-related deaths [33]. Sports and recreational activities are also a major cause of TBI. Sports related mild TBI does not always involve a loss of consciousness and can go unrecognized and uncounted. It has been estimated that 1.6 to 3.6 million sports related TBI occur every year and can cause long term cognitive problems [70]. Another major risk factor for TBI is military combat, particularly in recent times with warriors wearing Kevlar helmets and sustaining blast injuries from improvised explosives. In a survey of U.S. Army infantry soldiers, 15.2% reported blast injuries in which they suffered either a loss of consciousness or altered mental status during deployment; of note these individuals were at substantially higher risk for post-traumatic stress disorder [50].

Children between the ages of 0-4 and adults over the age of 75 years have the highest rate of TBIs [10]. Males appear to have more TBIs, with a higher rate of hospitalization, emergency visits and deaths related to TBI than the female population [33] who appear to be protected by the hormone progesterone [2,27].

1.2. Clinical classification

A TBI is defined as an injury to the brain, caused either by blunt trauma, penetrating trauma, rapid motion or blast injury to the cranium which leads to a disruption of regular cerebral function [96]. TBI can range from mild to severe, and while there are multiple ways to classify TBIs, they all suffer from limitations. Currently, the most common scoring system for severity of brain injury is the Glasgow Coma Scale (GCS), which classifies injuries as mild, moderate or severe, by scoring the level of consciousness at first medical contact after a head trauma [73]. The score is the sum of three physical exam findings:

eye, motor and verbal reflexes. A GCS score of 13, 14 or 15 defines "mild TBI", typically a brief change in mental status or consciousness, and interchangeably called "concussion". "Moderate TBI", with GCS score of 9-12 [73], predicts a higher probability of permanent disability. GCS scores of 3-8 define "severe TBI", typically presenting with an extended period of unconsciousness and coma, and predict a high risk of death [73]. Other similar scoring systems based on clinical findings include the injury severity score, Brussels coma scale, Grady Coma Scale, and Innsbruck coma scale.

After neuroimaging (typically, non-contrast computed tomography scan of the brain), brain trauma is described clinically by anatomic location and pattern of injury. Severe TBI can be heterogenenous, and there are at least 6 different patterns of injury that can result in GCS < 8 [96]. Patterns include subdural hematoma, cerebral contusion, or subarachnoid, intraparenchymal, or intraventricular hemorrhage, with or without brain edema or "midline shift" [96]. Diffuse axonal injury without bleeding can also result in coma and death, but can only be seen on magnetic resonance imaging (MRI). Severity classifications can also be made according to the patho-anatomic classification of injury, describing the location, anatomical features of injury and size of lesion. The Marshall Score is based on CT scan results, and can be used to predict the risk of increased intracranial pressure and outcome in adults [74].

There are several clinical tools that are used to measure clinical outcomes in patients who survive TBI. The Glasgow outcomes score (extended), and disability rating scale, have been used to describe clinical outcomes or neurologic injury severity [74]. Unfortunately, there are currently no diagnostic tools that can accurately predict outcomes

after a TBI. Current methods of classifying TBI do not take into account the subsequent secondary systemic effects that may occur during the recovery period following TBI.

1.3 Pathophysiology

TBI is a heterogeneous disease that has variable pathophysiological outcomes which compound the issue of diagnosis and treatment. As described in the section above, injury can be classified by the physical mechanism of impact (blunt, penetrating, rotational, or blast), pathoanatomic features, and clinical findings. Primary injury to brain structures by any mechanism happens immediately and is a direct result of mechanical trauma [73]. Tissue damage may include shearing of white matter, focal bleeding, swelling within gray matter, hematomas, microporation of membranes, and vascular injury [60,74]. The physical mechanism of impact can predict the type and severity of the tissue injury. Different mechanisms such as direct impact, rapid acceleration or deceleration, penetration (gunshot) or blast waves from an explosion produce typical patterns of tissue damage [74]. Diffuse axonal injury, with multiple lesions or shearing of white matter tracts, occurs most commonly through an acceleration/deceleration mechanism [52].

Secondary insults after TBI can arise hours to days later and may involve the release of neurotransmitters (glutamate), ion channel leakage, reactive oxygen species (ROS) production, blood brain barrier (BBB) breakdown, edema and increased intracranial pressure (ICP) [31,73,74,82]. Progression of intracranial hemorrhage and progressive edema are the most common secondary insult following an initial traumatic injury [96]. Common secondary changes that occur to the brain after TBI also include progressive

white matter changes (after axonal injury), reactive gliosis, and degeneration of neural tissue and Tau protein accumulation, which has been associated with chronic traumatic encephalopathy [13]. The most common form of TBI is diffuse axonal injury where there are multiple lesions or shearing of white matter tracts and occurs most commonly through acceleration/deceleration impact such as a car accident [52]. Although the primary brain injury is untreatable from the standpoint of the clinician, secondary effects of TBI may be avoidable and are of major interest to researchers and health care providers.

The cerebral circulation appears highly vulnerable to secondary pathophysiological effects after TBI. One of the most important physiological properties of the cerebral circulation is cerebral autoregulation which is the mechanism that maintains cerebral blood flow (CBF) constant despite changes in cerebral perfusion pressure [91]. Cerebral autoregulation is maintained by myogenic, neurogenic, and metabolic mechanisms. The myogenic component is defined as the inherent mechanism of vascular smooth muscle cells (VSMCs) to constrict or dilate in response to changes in intramural pressure [91]. Multiple studies have demonstrated that more than 50% of TBI patients have shown an impairment in autoregulation [31,40,55,91]. Disrupted autoregulation is often associated with a high mortality rate and poor outcome in TBI survivors, even in minor brain injuries [96]. The loss of cerebral vascular autoregulation increases the susceptibility of harmful secondary effects such as hypoxia, ischemia and infarction [49,91]. Time course studies show that within 3-5 days post-injury, over 80% of patients display an impaired autoregulation [105]. Patients who were unable to reestablish a normal autoregulation after two weeks had unfavorable outcomes that correlated with a low GCS score, diffuse brain injury and

elevated ICP [105]. Changes in cerebral vascular tone and vasodilation are due at least in part to changes in endothelial NO production [111]. The loss of vascular compensation in response to reduced cerebral blood flow can result in regional ischemia and often results in death. TBI can also have effects on the permeability properties of the cerebral vessels, contributing to edema [102].

The BBB which is responsible for maintaining homeostasis between blood circulation and the central nervous system could be compromised in TBI [102]. This breakdown may last from several days to weeks or even years after injury occurs and the extent of damage is directly related to the method of injury [107]. Primary damage to the brain can lead to injured endothelial cells, leaky channels, a loss of vascular integrity and metabolic processes [95]. BBB breakdown may occur as a result of initial injury or secondary effects such as inflammation or metabolic disturbances. Secondary brain damage that arises from BBB breakdown includes increased endothelial permeability which leads to brain edema [102]. Edema that emerges from TBI can lead to fluid accumulation in the brain and can lower perfusion of cerebral blood flow, exacerbating detrimental effects to the TBI survivor [73,102].

Along with cerebral complications, systemic complications can also arise from TBI such as hypertension and coagulopathy [62,118]. Many of the systemic effects seen after TBI including increased blood pressure, cardiac output, deregulated temperature, metabolic changes, and oxygen utilization are associated with increased levels of circulating catecholamines [18,19,51]. Studies have shown that there is an increased sympathetic discharge, with a large amount of catecholamines release (norepinephrine,

epinephrine, and dopamine) in circulation following TBI; the amounts of circulating catecholamines present correlates with a lower GCS and more severe injury [19]. Sustained sympathetic hyperactivity peaking 1-3 weeks after injury and elevated circulating catecholamines also predict poor outcome and increased mortality rate [18,19,46,93]. Some individuals who sustain a TBI may develop a rare and severe complication called a paroxysmal sympathetic hyperactivity syndrome "sympathetic storm" [88]. Patients who suffer from sympathetic storm experience bouts of tachycardia, hypertension, sweating and increased respiration that often occurs in waves and correlates with an elevation of ICP [88]. The pathophysiology of sympathetic storm requires further study, but is likely related to damage to parts of the brain that control autonomic nervous system functions.

The sympathetic hyperactivity response from TBI can mediate cardiovascular abnormalities and myocardial dysfunction [41,45]. These abnormalities lead to increased blood pressure, increased pulse, tachypnea, cardiac contractility, increased troponin levels and abnormal ECGs which may contribute to mortality [15,71,75]. In patients who endure a head injury, 20-30% with subarachnoid hemorrhage will subsequently suffer from cardiomyopathy, this has been referred to as neurogenic stress cardiomyopathy (NSC) [15]. Patients with NSC are at particularly high risk for potentially fatal complications such as ventricular arrhythmias and SAH-associated cerebral vasospasm. ECG abnormalities are observed in 75-92% of patients with intracranial bleeding [15]. Cardiovascular effects of TBI has been described in a rat model where they reported myocardial damage from TBI; myocytolysis, contraction band necrosis, edema formation and interstitial mononuclear cell

infiltration was seen in cardiac myocytes taken at different time periods following TBI [85]. In the 24 hour trauma group, TEM analysis of cardiac myocytes showed swollen mitochondria, dilated sarcoplasmic reticulum and large edematous areas of the cytoplasm with significantly levels of nuclear damage [85]. Sympathetic release of catecholamines may cause cellular stress and damage by generating ROS and forming oxidation products [92]. ROS have also been reported in cardiac tissue in a TBI rat model produced by our laboratory [71]. TBI affects not only the central area of impact but can also damage peripheral organs such as the cardiovascular system resulting in cellular damage and increased mortality.

There have been numerous clinical trials for TBI over the years including corticosteroids [67], hypothermia [6], and progesterone [113], but few treatment options have been shown to decrease the secondary effects of TBI. One promising area of research is with beta adrenergic receptor antagonists (β-blockers) for treatment of TBI [4]. Adrenergic receptor antagonists may be used to try to control the sympathetic hyperactivity that follows TBI, and can reduce the impact of sympathetic stimulation by regulating hypertension and cardiac output [4]. In a large retrospective review, TBI patients who happened to be on β-blockers prior to their head injury had a statistically decreased mortality rate compared to those who were not [20]. In another study of TBI survivors with a GCS of 5, treatment with propranolol lowered their increased metabolic expenditure and hypertension [93]. Decreasing TBI-induced systemic hypertension could protect TBI survivors from developing cerebral edema and improve cerebral autoregulation. However, prospective studies of adrenergic receptor blockade for TBI are needed to determine if

these drugs are a suitable treatment to improve long-term outcome. Despite the extensive literature on secondary cardiovascular effects after TBI, there are no studies focusing specifically on effects of TBI in systemic blood vessels and particularly the endothelium.

1.4 Endothelial Vasodilatory Functions

The endothelium is a single layer of cells which lines the intraluminal space inside of blood vessels. The endothelium has specialized tight junctions, serving as a physical barrier between circulating blood cells and the extravascular or interstitial space. More than just providing a physical barrier, the endothelium plays a larger role in cardiovascular physiology; it modulates inflammatory responses, coagulation, angiogenesis, and vessel diameter. Small resistance sized arteries with diameters less than 200 microns play a critical role in the regulation of peripheral vascular resistance and dysregulation of vascular tone which may contribute significantly to high blood pressure [63]. The focus of this section will be specifically on the multiple factors released by the normal endothelium in resistance size arteries that contribute to vasodilation and/or vasoconstriction processes. The role of the endothelium in relaxation of blood vessels has been extensively discussed and reviewed since its first appearance in the literature in 1980 by Furchgott [39]. It is now recognized that there are two main pathways involved in endothelial-dependent dilation of VSM, nitric oxide (NO) and endothelial dependent hyperpolarization (EDH). The products of cyclooxygenase activity also dilate some vascular beds but this pathway does not appear to play a role in dilation of resistance-sized arteries and therefore is not reviewed in this paper.

1.4.1 Nitric Oxide

The involvement of NO in vascular function was first described by Furchgott and Zawadski in 1980 and was initially called "endothelial derived relaxing factor" or EDRF [39]. EDRF was shown to be released by the endothelium upon activation by ACh; sodium nitroprusside (SNP), glyceryl trinitrate and NO mimic the response to ACh on the muscarinic receptor. It was determined later that NO and EDRF were the same species, shown by similar concentration curves and decay rates [86].

NO is a vascular protective chemical, i.e. it protects from accumulation of platelets and plaque formation, inhibits proliferation of VSMC and decreases expression of genes that engage in the proinflammatory response [38]. Endothelial cells synthesize NO by endothelial nitric oxide synthase (eNOS), a Ca^{2+}-dependent isoenzyme that may be constitutively active, but is primarily activated by G-protein coupled receptor activation or by shear stress induced by increased blood flow. This in turn activates eNOS through a Ca^{2+}-dependent mechanism [35]. eNOS converts L-arginine to L-citrulline releasing NO, in the presence of the cofactors Tetrahydrobiopterin (BH_4), oxygen, Flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH) [38]. NO diffuses into the VSM and activates soluble guanylyl cyclase (sGC) which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP), activating cGMP-dependent protein kinase (PKG) and promoting vasodilation. PKG has multiple targets in the VSMC. PKG general targets include 1) L-type Ca^{2+} channels on the plasma membrane, which causes them to inactivate, 2) phospholamban on the sarcoplasmic reticulum (SR) releasing its inhibition of the sarcoplasmic reticulum Ca^{2+} ATPase and thereby increasing

Ca^{2+} uptake from the cytosol, 3) IP_3 receptors on the SR which inhibit release of Ca^{2+}, and 4) VSM large conductance Ca^{2+} activated K^+ channels (BK_{Ca}). These events all lead to decreased intracellular calcium $[Ca^{2+}]_i$ and vasodilation [23,36-38,42].

1.4.2 EDHF in the vascular endothelium

Endothelial-dependent hyperpolarization factor (EDHF) is an endothelial function that can hyperpolarize and subsequently relax VSMC independently of NO and prostacyclins. It involves the endothelium, and incorporates specifically endothelial Ca^{2+}-activated potassium channels (K_{Ca}) [14]. There is a variation in the nature and mechanisms of EDHF among different vascular beds and species. EDHF can be activated by agonists such as ACh, bradykinin, histamine or substance P causing hyperpolarization and subsequent VSMC relaxation by decreasing cytosolic Ca^{2+} [68]. The EDHF-mediated pathway in rat mesenteric arteries involve K_{Ca}, specifically: small conductance (SK_{Ca}) and intermediate conductance (IK_{Ca}) potassium channels [14,30,62,68,77]. SK_{Ca} and IK_{Ca} channels have a Ca^{2+} dependent relationship that is mediated by calmodulin, and lack voltage sensitivity. In experimental preparations, the EDH component of ACh-induced dilation can be evaluated using pharmacology to block K_{Ca} with charybdotoxin (BK_{Ca} and IK_{Ca} channel blocker) and apamine (SK_{Ca} channel blocker) [68]. Of note, apamine and iberiotoxin (BK_{Ca} channel blocker) are unable to prevent SMC hyperpolarization and ACh-induced vasodilation [26]. This finding introduced confusion until it was later discovered that while apamin and iberiotoxin together can completely abolish SK_{Ca} and BK_{Ca} channel ion flow, neither can block IK_{Ca} channels. Charybdotoxin is thus used to block not only

BK_{Ca} but also IK_{Ca} channels. Together, SK_{Ca} and IK_{Ca} channels in the endothelium appear to account for the EDH component of ACh induced vasodilation.

ACh activates muscarinic receptors on EC surface and induces a release of Ca^{2+} from the ER through IP_3 channel activation. As a result, increased $[Ca^{2+}]_i$ triggers SK_{Ca} and IK_{Ca} (and also eNOS) to cause efflux of K^+ out of the EC and into the myoendothelial space. The increased concentration of K^+ in the myoendothelial space activates VSMC internally rectifying potassium channels (K_{IR}), causing VSMC hyperpolarization and relaxation [34]. Hyperpolarization of VSMC deactivates voltage dependent calcium channels (VDCC), reducing Ca^{2+} influx and Ca^{2+} affinity of contractile proteins for Ca^{2+}.

Recent results indicate that in mouse mesenteric arteries the transient receptor potential vanalloid 4 (TRPV4) channels plays an important role in amplification of the ACh response [103]. Hyperpolarization of the endothelium elicits TRPV4 mediated influx of extracellular Ca^{2+}. TRPV4 channels are the predominant extracellular Ca^{2+} influx pathway involved in the vasodilation of mouse mesenteric arteries in response to ACh and contributes approximately 75% to VSMC hyperpolarization and vasodilation [28]. Activation of few TRPV4 can lead to maximal vasodilation, in part due to the localization of these signals at microenvironments in the myoendothelial projections. Increases of $[Ca^{2+}]_i$ through activation of TRPV4 also stimulate BK_{Ca} channels through ryanodine receptor mediated Ca^{2+} sparks inducing vasorelaxation [28].

Endothelial cell hyperpolarization may be transferred to the VSMC via gap junctions within these myoendothelial projections, as suggested by the fact that inhibition

of gap junctions by anti-connexin antibody decreases endothelial-dependent relaxation [30]. Microdomain environments exist between SK_{Ca} and K_{IR}, and IK_{Ca} and K^+/Na^+-ATPases [30] and can contribute to the cooperation of K_{Ca} channels to maintain a balanced response to shear stress, receptor agonists and electrochemical changes in the cell.

Epoxyeicosatrienoic acids (EETs) are metabolites of arachidonic acid through the CYP enzymatic pathway and are candidates for EDHF. ETTs are synthesized in endothelial cells and cause hyperpolarization of VSMC through the activation of large conductance BK_{Ca} channels [63]. Previous studies show that mouse mesenteric arterioles rely on TRPV4-mediated activation of the EETs pathway to cause hyperpolarization and subsequent vasodilation of resistance arteries [68]. EETs-induced vasodilation can be inhibited by ChTx, the BK_{Ca} IK_{Ca} channel blocker, suggesting its role in the EDHF pathway [68,80].

Hydrogen peroxide (H_2O_2) has also been reported to cause relaxation in vascular tissue depending on the arterial bed. H_2O_2 can cause vasorelaxation in mouse mesenteric arteries and may act as an EDHF [29]. This was shown in small mouse mesenteric artery dilation where ACh induced hyperpolarization was abolished with incubation of catalase in the absence of NO and COX derived pathways [78].

The contribution of EDHF to vasodilation has been extensively studied, and the specific factors driving hyperpolarization vary in different vascular beds and diameter sizes [30,39,62,68,99].

1.5 Endothelial Dysfunction

Endothelial dysfunction was first described by Furchgott and Zawadski when they observed an attenuated response to ACh after accidently rubbing the intraluminal layer of a rabbit aorta [39]. They concluded that arterial relaxation by ACh required the presence of endothelial cells [39]. Endothelial dysfunction has been described in a variety of vascular related diseases such as diabetes [97], atherosclerosis [65], ischemia-reperfusion [77], and hypertension [63]. Endothelial dysfunction is typically defined as an impaired vasodilatory response to the muscarinic receptor agonist, ACh. Mechanisms that impair the vasodilatory function of the endothelium include oxidative stress, impairment of NO production (through eNOS uncoupling, increased arginase activity, or depletion of tetrahydrabiopterin), and impairment of EDHF induced-hyperpolarization. Evidence for each of these mechanisms in endothelial dysfunction is provided below.

1.5.1 Reactive oxygen species (ROS)

Oxidative stress and reactive oxygen species (ROS) have been common variables in endothelial dysfunction. ROS are produced under normal conditions in the cell through multiple enzymatic pathways such as eNOS, COX, lipoxygenase, cytochrome P450, and NADPH oxidases [38]. Disruption to theses enzymes can lead to uncoupled behavior resulting in an increase production of ROS [83]. NADPH oxidase has been implicated in the production of the superoxide anion (O_2^-), which in turn reacts with eNOS-derived NO to form peroxynitrite ($ONOO^-$). $ONOO^-$ has been shown to cause damage to endothelium through peroxidation of lipids, sulfhydryl groups, tyrosine groups

and guanine groups [94]. Accumulating amounts of $ONOO^-$ can uncouple eNOS by oxidizing the Zn-thiolate in the enzyme and its cofactor tetrahydrobiopterin (BH_4), dissociating the monomers, reducing NO bioactivity and uncoupling eNOS [94]. NOS in an uncoupled state results in an electron transfer to oxygen instead of its substrate L-arginine, creating ROS [83]. Formation of $ONOO^-$ has also been shown to inhibit sGC and inactivate prostacyclin synthase by tyrosine nitrosylation [38,94,120].

ROS, particularly O_2^- are believed to contribute to the pathophysiology of hypertension by reducing the bioavailability of NO. ROS have also been implicated as a mechanism of cerebrovascular dysfunction following TBI [6]. ROS also act on vascular ion channels, including VSM L-type Ca channels and endothelial SK channels [3,16]. ROS production in vascular disease can inhibit all three of the primary endothelium-dependent vasodilator pathways (NO, EDH and prostacyclin) and tilts the balance of the endothelium towards constriction.

1.5.2 Uncoupling of eNOS

Under pathological conditions, eNOS can act in an uncoupled mode producing oxygen radicals that contribute to vascular diseases such as diabetes [100], hypertension [69], atherosclerosis [101], and obesity [116]. The "uncoupled" eNOS enzyme donates electrons to O_2 instead of L-arginine, thereby producing superoxide rather than its usual product NO. $O_2^{-\cdot}$ can react with NO producing peroxynitrate ($ONOO^-$) which decreases NO bioavailability [94]. Oxidation of the eNOS zinc-thiolate cluster by $ONOO^-$ can uncouple eNOS [38]. In a study conducted on DOCA-salt treated hypertensive mice, ROS

production was lowered by inhibition of NOS and knocking down nicotinamide dinucleotide phosphate oxidases (NOX), suggesting that NOX activity produces ROS and uncouples eNOS [69].

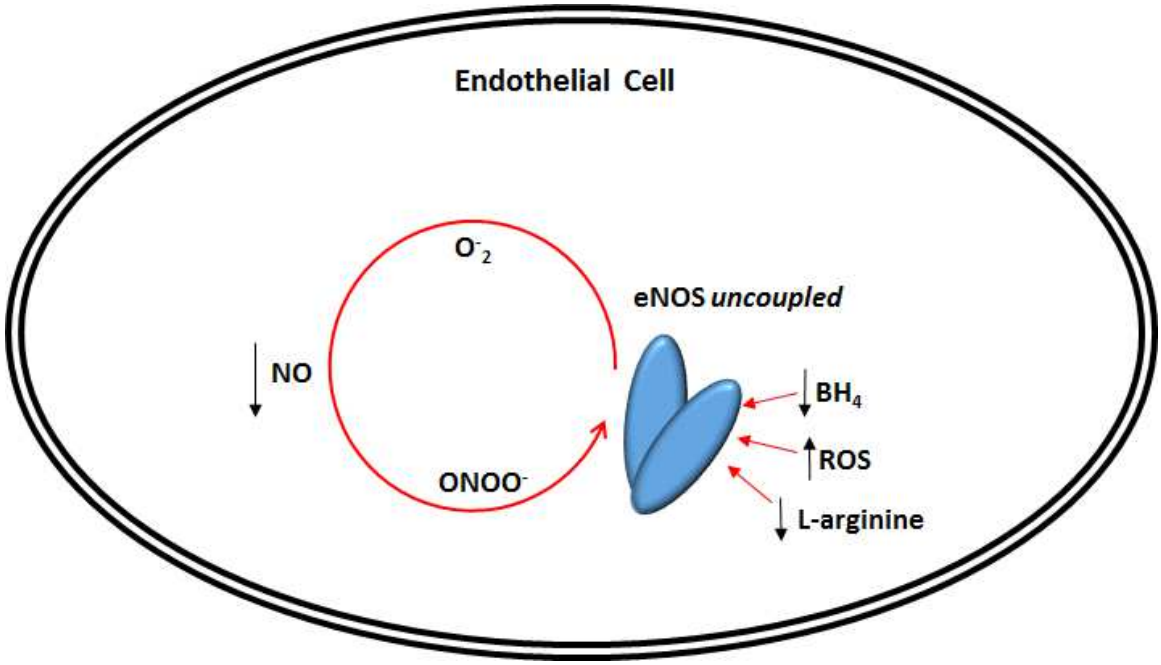

Figure 1 Schematic of eNOS uncoupling: Endothelial nitric oxide synthase (eNOS) can become uncoupled through multiple factors such as a decreased amount of its cofactor BH_4, increased reactive oxygen species (ROS) and decreased amounts of its substrate L-arginine. In an uncoupled state eNOS produces superoxide (O_2^-) anions which then scavenge NO, producing peroxynitrite molecules which further uncouple the enzyme. Decreased NO can then lead to increased vasoconstriction of the artery.

1.5.3 Role for L-Arginine and arginase in eNOS uncoupling

L-arginine is the rate-limiting substrate in the enzymatic production of NO through eNOS. Decreased L-arginine has been shown to contribute to eNOS uncoupling and lead to disruption of endothelial-dependent vasodilatory function [53]. Under pathological conditions, L-arginine supplementation has been shown to restore endothelial dependent dilation [53]. In example, it has been demonstrated that ACh-induced vasodilations are decreased after successive concentration response curves were performed on the same artery [53]; this attenuated response to ACh on the 2nd and 3rd treatment is due to the depleted stores of endothelial derived L-arginine which can be rescued by supplementation of L-arginine [53].

Arginase is a major consumer of L-arginine and can compete for L-arginine in the vascular endothelium, uncoupling eNOS [38]. Increased arginase activity has been reported to contribute to endothelial dysfunction in multiple diseases such as aging [8], ischemia–reperfusion [47], hypertension [57], atherosclerosis [115], diabetes [101] and obesity [44]. These studies show that arginase plays an important role in regulating NO bioavailability by competing with eNOS for L-arginine [8,44,47,57,101,115]. Arginase inhibition can help restore dysregulated endothelial function by increasing eNOS dependent NO production in the endothelium. A specific inhibitor of arginase, nor-NOHA, an analog of an intermediate in the conversion from L-arginine to NO which interrupts its Mn-cluster active site [21] has been shown to rescue dysfunctional endothelial-dependent vasodilations in arteries [44,101].

In a diet induced obesity model, mouse aortas from high fat diet mice showed an increase in arginase expression and a decreased expression of eNOS when compared to

low fat diet mice aorta [44]. Inhibition of arginase by nor-NOHA restored NO levels in the aorta of high fat diet mice [44]. Rescue of endothelial-dependent vasodilations have also been shown in patients diagnosed with type-II diabetes mellitus and coronary artery disease who displayed elevated levels of arginase expression [101]. Infusion with the specific arginase inhibitor nor-NOHA restored vasodilatory responses to SNP and also increased eNOS expression in vascular tissue [101]. Supplementation of L-arginine or inhibition of the enzyme arginase can restore NO production in multiple disease states, providing a potential therapeutic target for cardiovascular disease.

1.5.4 Tetrahydrobiopterin (BH_4) and eNOS uncoupling

A critical determinant of eNOS activity is the availability of its cofactor (BH_4) [110]. Uncoupling of eNOS has been implicated in a number of diseases with decreased BH_4 levels, including atherosclerosis, diabetes and hypertension [24,25,101]. Deficient BH_4 availability in the endothelium has been correlated with decreased NO bioavailability, impaired cGMP, and vasoconstriction [110]. Under conditions of limited BH_4 availability (due to oxidation or reduced formation), eNOS functions in its "uncoupled" state and NADPH-derived electrons are added to molecular oxygen instead of L-arginine, leading to the production of superoxide.

BH_4 is a reducing agent and can become a target for oxidation during cellular oxidative stress which in turn lowers its bioavailability. Low levels of BH_4 further contribute to an oxidative state which has been seen in high fructose-fed rats and hypercholesterolemic patients [110], and has been linked to hypertension [69]. Oral and

intravenous supplementation of the eNOS cofactor BH_4 has been shown to lower intracellular levels of ROS and rescue ACh-induced endothelial-dependent vasodilation in vascular disease models [69,89,100].

Supplementation of BH_4 can be performed by introducing the BH_4 precursor sepiapterin [100]. Sepiapterin can be reduced in all cells by sepiapterin reductase to 7,8-dihydrobiopterin (BH_2) and further by dihydrofolate reductase to form BH_4 by the salvage pathway [84]. In obese mice, BH_4 supplementation with sepiapterin rescued vasodilation in response to ACh, which was blocked by L-NAME [89]. Importantly, BH_4 supplementation in animals with these diseases improves endothelial dysfunction [110].

1.5.5 EDHF

EDHF is also thought to contribute to vascular dysfunction, in rat models of diabetes [72,112] and hypertension [64]. In a study in cultured human kidney cells, ROS could impair EDHF hyperpolarizations by directly inhibiting the function of BK_{Ca} channels in the VSMC by modifying a cysteine residue on BK_{Ca} channels and decreasing its sensitivity to Ca^{2+} [106]. The EDHF component of vasodilation can become altered in disease states and can lead to dysfunctional vasodilations in response to agonist stimulation. In streptozotocin-induced diabetic rats, the role of EDHF and NO-type relaxations in mesenteric arteries was impaired shown by decreased ACh-induced vasodilation in the presence of NO inhibitors and EDHF-associated channel blockers [72]. In an angiotensin infusion induced hypertension model, ACh-induced vasodilations in mesenteric arteries resulted in attenuated responses compared to controls [64]; in that

study, inhibition of NOS with L-NAME completely abolished ACh-dilations in hypertensive mesenteric arteries, implying that the EDHF pathway was dysfunctional [64].

1.6 Myogenic Tone in Resistance Arteries

The myogenic response is the intrinsic ability of the VSMC to constrict or dilate in response to changes in intravascular pressure [5,91]. The myogenic response was first described in 1902 by Bayliss, when he noted that excised arteries would contract to increased intramural pressure and relax under lesser pressure independent of the peripheral nervous system [7]. Previous studies have shown that the strength of myogenic tone varies throughout vascular beds and can also be dependent on artery size in the same vascular bed [98].

Mechanical stretch induced by increasing intraluminal pressure activates non-selective stretch activated ion channels to elicit VSMC depolarization [98]. Changes in membrane potential in turn activate L-type calcium (Ca^{2+}) channels, leading to influx of Ca^{2+} through these channels increasing intracellular Ca^{2+} ($[Ca^{2+}]_i$) [48]. Rising $[Ca^{2+}]_i$ activates myosin light chain kinase (MLCK), which can then phosphorylate myosin light chain (MLC) allowing it to interact with ATP and bind to actin to contract VSMC. Myosin light chain phosphatase (MLCP) removes the phosphate group from MLC inhibiting its interaction with ATP and prevents SMC contraction. This mechanism allows for the detachment and reattachment of myosin to actin causing vasoconstriction [98]. BK_{Ca} channels also play a role in myogenic tone by providing a negative feedback mechanism to oppose vasoconstriction. Increased $[Ca^{2+}]_i$ induces Ca^{2+} sparks, spatiotemporal increases

of cytosolic Ca^{2+} released by ryanodine receptors found on the sarcoplasmic reticulum (SR) [12]. Ca^{2+} sparks activate BK_{Ca} channels and hyperpolarize the cell, inhibiting further constriction [12].

Vascular tone can also function independently of increasing $[Ca^{2+}]_i$ levels. The ratio of MLCK and MLCP activity modulates MLC Ca^{2+} sensitivity; enzymes such as protein kinase C (PKC) or Rho-kinase (ROK) also contribute to this mechanism. Phosphorylation of MLCP by PKC or ROK can increase myogenic tone by inhibiting dephosphorylation of MLC, promoting SMC contraction.

It is generally accepted that the endothelium has a role in modulating tone by releasing basal amounts of vasodilators like NO to oppose vasoconstriction. The involvement of the endothelium in regulating tone can vary in different species and vascular beds [17,59,79]. For example, a study showed that rat mesenteric arteries removed of endothelium (denuded) experienced no change in myogenic tone in response to step-wise increase in intraluminal pressure [104]. In contrast, denuded cerebral arteries had a profound increase in myogenic tone due to an endogenous endothelial NO production [111]. In the mouse, denuded mesenteric arteries had enhanced myogenic response, which was attributed to the loss of endothelial-dependent NO release [99]. The ability to develop and maintain myogenic tone is imperative in small resistance arteries, where loss of function or modulation is often correlated with the development of disease.

CHAPTER 2: ENDOTHELIAL-DEPENDENT DILATIONS ARE IMPAIRED FOLLOWING TBI

2.1 Introduction

TBI is the most frequent cause of death in children and young adults and is increasingly recognized as a major health care problem in the United States [73]. Individuals who survive TBI may suffer from long term effects that will significantly lower the overall quality of life. TBI is a heterogeneous disease that has variable pathophysiological outcomes in the area of injury (the brain) and areas peripheral to injury.

Along with cerebral complications, systemic complications can also arise from TBI. Systemic effects induced by TBI include increased blood pressure, temperature imbalance, metabolic changes and increased oxygen utilization; all these effects can be correlated with an increased sympathetic discharge of circulating factors such as catecholamines [18,19,46,93]. Sympathetic hyperactivity induced by TBI has negative effects on the cardiovascular system, coagulopathy and acute lung injury [11,85,92,118].

However, beyond cardiovascular effects, there are no data showing peripheral damage to the vasculature caused by TBI-induced circulating factors. Arterial vasculature, especially small resistance arteries, plays an important role in regulating peripheral vascular resistance [60]. Increased vasoconstriction in resistance arteries may serve as a compensatory mechanism to maintain optimal circulatory efficiency and redistribute blood flow between and within organs to maintain normal levels of blood pressure [43]. Basal constriction (myogenic tone) of vascular smooth muscle cells (VSMC) and relaxation through endothelial-dependent pathways are key factors in the regulation of blood flow [4].

The endothelium, a single layer of cells which lines the intraluminal surface of vessels, releases vasoactive factors that contribute to vasoconstriction and vasodilation. In resistance arteries, there are two main endothelial-dependent pathways that lead to vasodilation: endothelial dependent hyperpolarizing factor (EDHF) and nitric oxide (NO). Dysfunctional endothelial-dependent pathways have been implicated in multiple vascular disease states such as diabetes, atherosclerosis, obesity, hypertension, aging, and many more [8,24,25,44,47,57,101]. Most notably, eNOS uncoupling acts as a significant contributor to vascular disease and impaired NO-production in the vascular endothelium. Under pathological conditions, eNOS can become uncoupled by increased ROS production in the vascular endothelium [38,69,94], reduced availability of its cofactor BH_4 [24,25,69,101,110] or increased expression of the enzyme arginase [38,47,57].

In this study, we investigated the effects of fluid percussion-induced TBI on endothelial-dependent vasodilatory functions in a remote systemic tissue bed (the mesenteric microcirculation), 24 hours after injury. Based on the literature reviewed here, we hypothesize that endothelial dysfunction, as defined by impaired endothelial-dependent vasodilation, occurs after a TBI, due to dysregulation of either the NO or EDH pathways of endothelial-dependent vasodilation.

2.2 Material and Methods

2.2.1 Animals

In this study, adult male Sprague-Dawley rats (300-350 g) were used to test the effects of TBI on mesenteric arteries. Experimental TBI was induced by a fluid percussion injury model as previously described [71]. Briefly, a craniotomy was performed in anesthetized rats and a bolt attached to a fluid filled column was placed between the bregma and lambda landmarks on the skull. Brain injury was induced by releasing a pendulum to create a pressure pulse that damages the brain. This method produces a mild and survivable brain injury keeping the dura intact and producing astrogliosis and neurological deficits [71]. Un-operated animals were used as controls for this study. After a 24hr recovery period the rats were anesthetized by an intraperitoneal injection (i.p) of sodium pentobarbital (30 mg/kg) and then euthanized by decapitation.

2.2.2 Isolation and pressurization of mesenteric arteries

After euthanasia, the small and large intestine with attached mesenteric tissue was placed in ice cold (4 °C) physiological saline solution (PSS of the following composition: NaCl 118.5 mM, KCl 4.7 mM, KH_2PO_4 1.18 mM, $MgCl_2$ 1.2 mM, $NaHCO_3$ 20 mM, $CaCl_2$ 2.5 mM, glucose 7.5 mM) and then pinned down on a sylgard plate. Fourth-order resistance mesenteric arteries (<200 μM in diameter) were chosen for this study because they displayed more myogenic tone compared to larger diameter mesenteric arteries (1^{st}-3^{rd} order) [22]. Arteries were isolated from surrounding tissue and were mounted on opposing small glass pipettes and secured with suture material in a small vessel chamber

(Living Systems Instrumentation, St. Albans, VT, USA). Vessels were briefly cannulated to remove intraluminal blood before experimentation. Arteries were pressurized using a pressure servo system (Living Systems Instrumentation, St. Albans VT, USA) to 80 mmHg with PSS induced by the proximal side of the mount and the distal end closed with a stop cock. Vessels were allowed to equilibrate for 15 minutes at low pressure (10 mm Hg) before pressure was raised to 80 mm Hg to allow vessels to develop spontaneous myogenic contraction. Endothelium integrity was tested in each vessel with the compound NS309 (1 μM) which increases Ca^{2+} sensitivity to SK_{Ca} and IK_{Ca} channels. Arteries that did not develop tone were discarded from the experimental series. The chamber was continuously superfused with warmed PSS (37°, pH 7.4) which was bubbled with bioair (75% Nitrogen, 20% Oxygen and 5% CO_2). The vessel chamber was placed on an inverted microscope with attached CCD camera and edge detection software (Ionoptix 6.0) to monitor lumen diameter changes. The Ca^{2+} channel blocker diltiazem (100 μM) in Ca^{2+}-free PSS was used to obtain passive diameter of vessels at the end of each experiment.

2.2.3 Assessment of endothelial dysfunction following TBI

ACh concentration response curves (CRC) were performed in mesenteric control and TBI arteries that spontaneously developed myogenic tone. Arterial responses were measured with increasing logarithmic concentrations of two endothelium-dependent vasodilators ACh (1 nM to 1 μM) and bradykinin (BK) (30 pM to 300 nM). To test the role of EDHF in mesenteric arteries following TBI, arteries were incubated with the NOS

competitive inhibitor N^G-nitro-L-Arginine (L-NNA, 100 μM) for 20-25 minutes prior to and during agonist treatment

2.2.4 Assessment of NO contribution following TBI

To test the role of NO in ACh induced vasodilation in TBI and control groups, EDHF antagonists apamine (30 μM) paxilline (1 μM) and charybdotoxin (200 nM)[103] were sequentially added to the superfusing bath for 10 minutes prior to ACh addition (1 μM). Apamine is a toxin derived from bees, and specifically inhibits SK_{Ca} channels. Paxilline is a peptide derived from penicillium which blocks BK_{Ca} channels. Charybdotoxin is obtained from scorpion venom and acts as a BK_{Ca} and IK_{Ca} channel blocker.

In order to obtain the contribution of K_{Ca} to myogenic tone, all blockers were added to the bath as follows: paxilline, charybdotoxin, apamine. Decrease in lumen diameter was calculated as a percentage of myogenic tone. ACh-induced dilation (1μM) was calculated in the presence of all three blockers as a percentage of passive to determine NO contribution.

2.2.5 Pressure-induced constriction (myogenic tone) and measurement of L-NNA induced vasoconstriction.

To determine whether TBI affects myogenic tone in mesenteric arteries, experiments were performed in pressurized arteries by increasing intraluminal pressure to 80 mmHg in control and TBI groups and quantifying % tone in comparison to maximal

diameter achieved under passive conditions (Ca^{2+}-free PSS and diltaiazem). To study the role of the endothelium in myogenic response in this particular vascular bed, arteries were endothelium denuded; endothelium was disrupted by either introducing 1-2 mL air into the lumen of the arteries for 5 minutes or mechanical damage by carefully passing a hair through the vessel. Removal of endothelium was determined by the lack of response to the SK_{Ca}/IK_{Ca} channel activator NS309 (1μM). Only arteries with <15% dilation to NS309 were considered as endothelium denuded.

2.2.6 Sepiapterin Supplementation

TBI and control arteries were incubated with L-sepiapterin (1 μM), a substrate for BH_4 synthesis through the salvage pathway, separate from the *de novo* pathway of BH_4 synthesis. Control and TBI vessels were incubated with sepiapterin for 30 minutes prior to a CRC of ACh was performed. Furthermore, the effect of sepiapterin on myogenic tone was also calculated in each experiment.

2.2.7 Arginase inhibition and L-Arginine supplementation

To rescue ACh induced vasodilations following TBI, mesenteric arteries from control and TBI animals were incubated with the specific arginase inhibitor Nω-hydroxy-nor-Arginine (Nor-NOHA; 20 μM) or with the NOS substrate N^G-amino-L-Arginine (L-Arginine; 100μM) for 30 minutes or until vessel diameter was stabilized; then, increasing concentrations of ACh (1nm to 1μM) were added in the presence of Nor-NOHA or L-Arginine.

2.2.8 Smooth muscle sensitivity to NO donors and contractility of vascular smooth muscle

To test the sensitivity of VSMC to NO after TBI in mesenteric arteries, the broad spectrum NOS inhibitor L-NNA (100 μM) was superfused for 20 minutes or until the vessel diameter was stabilized, followed by increasing logarithmic concentrations of the NO donor spermine NONOate (1 nM to 1μM).

SMC contractility was tested in control and TBI mesenteric arteries by introducing logarithmic concentrations of the analog of the endoperoxide prostaglandin H2 U46619 (1nM to 1μM).

2.2.9 Drugs and Reagents

NS309 (6,7-Dichloro-1H-indole-2,3-dione 3-oxime) was purchased from Tocris (Minneapolis, MN), 100 mM stock aliquots diluted with DMSO were prepared prior to experimental series and were stored at -20°C. ACh [2-(Acetyloxy)-N,N,N-trimethylethanaminium chloride] and L-NNA [N5-(Nitroamidino)-L-2,5-diaminopentanoic acid] were purchased from Sigma-Aldrich (St. Louis, MO). ACh aliquots were diluted with Milli-Q water and stored at -20 °C and discarded after 1 month. L-NNA was stored in powder form at room temperature; dilutions were made in 1 M HCl and kept at room temperature. Charybdotoxin was purchased from International Peptides and was diluted with MilliQ water. Apamine and paxilline were purchase from Sigma-Aldrich (Saint Louis, MO). Aliquouts were made in either DMSO or milliQ water and were stored at -20 °C. L-sepiapterin 2-amino- 7,8-dihydro-6-(2S-hydroxy-1-oxopropyl)-

4(1H)-pteridinone was purchased from Cayman Chemical (Orlando, Florida) and stored in powder form at -20°C. Fresh dilutions in DMSO were prepared immediately before vessel incubation. Nor-NOHA was purchased from Cayman Chemicals (Ann Arbor, MI) and was diluted with DMSO and stored at -20°C. L-Arginine was purchased from Sigma-Aldrich (Saint Louis, MO), dilutions were made with milliQ water and was stored at -20°C. The NO donor, Spermine NONOate [(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate and U-46619 were purchased from Cayman Chemicals (Ann Arbor, MI). Serial dilutions of spermine NONOate were made with .01 M NaOH and were stored at -20°C, and U-46619 dilutions were made in DMSO and stored at -20°C.

2.2.10 Analysis and Statistics

Myogenic tone was calculated as the percentage (%) decrease of the lumen diameter in comparison to Ca^{2+} free PSS in the following equation: Tone (%) = $[(D_{passive}-D_{active})/D_{passive}]x100$, where $D_{passive}$ = the (passive) lumen diameter of the artery in Ca^{2+}-free PSS containing the vasodilators diltiazem (100 μM) and D_{active} = the (active) lumen diameter of the artery in Ca^{2+}-containing PSS.

% Vasodilation was calculated as the difference in diameter before and after agonist administration normalized to maximum diameter in Ca^{2+} free PSS (D_{drug} - $D_{baseline}$ / $D_{passive}$ - $D_{baseline}$)x100. % Vasoconstriction was calculated as the difference in diameter before and after charybdotoxin, paxilline, apamine, and U46619 administration normalized to the baseline diameter before addition of drug (D_{drug} - $D_{baseline}$ / $D_{baseline}$)x100.

Values are represented as means ± standard error mean (SEM). Concentration-response curves were calculated with GraphPad Prism software (version 6.03; GraphPad Software, Inc) to fit the Hill slope from the data (variable slope model) for each individual experiment and then averaged to obtain the mean and SEM values. Statistical comparisons were performed using GraphPad Prism. Because of generally small sample sizes (example n=5 to 12), the nonparametric test Mann Whitney was used for comparison between 2 groups. We also used repeated measures analysis of variance (ANOVA) for the comparison of multiple groups at different concentrations. Statistical significance was considered at the level of $P<0.05$.

2.3. Results

2.3.1 Agonist-induced vasodilations in mesenteric arteries from control and TBI animals

Measurement of the extent of vasodilation induced by the muscarinic-receptor agonist ACh is the standard assay for endothelial dysfunction in pressurized arteries. To assess endothelial function following TBI, ACh concentration-response curves (1 nM to 1 μM) were performed in fourth order mesenteric arteries isolated from control and TBI animals and constricted with myogenic tone (Figure 2A). ACh-induced vasorelaxation was significantly attenuated in arteries from TBI rats compared to control (Figure 2C) suggesting a compromised endothelial-dependent vasorelaxation following TBI (n= 6, $P<0.0001$, Two-way ANOVA). Maximal dilation to ACh (1 μM) was severely diminished in TBI arteries compared to controls (59 ± 9% of passive diameter, n=6 *vs* 100 ± 1% of

passive diameter, n=6)). $LogEC_{50}$ values for control and TBI arteries were -7.4 ± 0.09 (n=6) *vs* -7.2 ± .43 (n=6), respectively, demonstrating a shift in the PD_2 values in TBI. Arteries from TBI animals could not maximally dilate to ACh, E_{max} was impaired.

In order to confirm the finding of endothelial dysfunction, and not simply downregulation of muscarinic receptors, we also performed concentration response curves with a different endothelial-dependent vasodilator bradykinin, which acts through bradykinin receptors. We performed a concentration-response curve of bradykinin (30 pM to 300 nM) in control and TBI arteries. Bradykinin (BK) induced vasodilations were also attenuated following TBI (n=5, Two-way ANOVA, $P<0.0001$). With a shift in the sensitivity to BK $LogEC_{50}$ values for control and TBI arteries were -9.0 ± 0.07 (n=5) vs -8.1 ± 0.04 (n=5), respectively. Arteries obtained from TBI animals dilated maximally to BK. These data confirm that endothelial-dependent vasodilations are impaired in mesenteric arteries from TBI rats.

2.3.2 EDHF contribution to ACh-induced vasodilation following TBI

To determine which endothelial derived pathway was causing a lack of vasodilation in response to ACh, we investigated the role of both EDHF and NO in ACh-mediated vasodilation. We evaluated the EDHF contribution by blocking the NO pathway with a broad NOS competitive inhibitor, L-NNA (100 μM) prior to and during ACh concentration challenge (1 nM to 1 μM) in both control and TBI arteries. In the presence of L-NNA (100 μM), ACh-induced dilations were attenuated in controls (1 μM ACh, 58 ± 12% of passive diameter, n=7, $P<0.0001$, Two-way ANOVA) but not in the TBI group (1 μM ACh, 63 ±

8% of passive diameter, n=6, Two-way ANOVA, n.s.) (Figure 2D), suggesting the EDHF component is intact in mesenteric arteries following TBI. These data indicate that the contribution of NO to ACh-induced dilations is blunted after TBI, suggesting either a lack of NO production or efficacy in mesenteric arteries obtained from TBI rats.

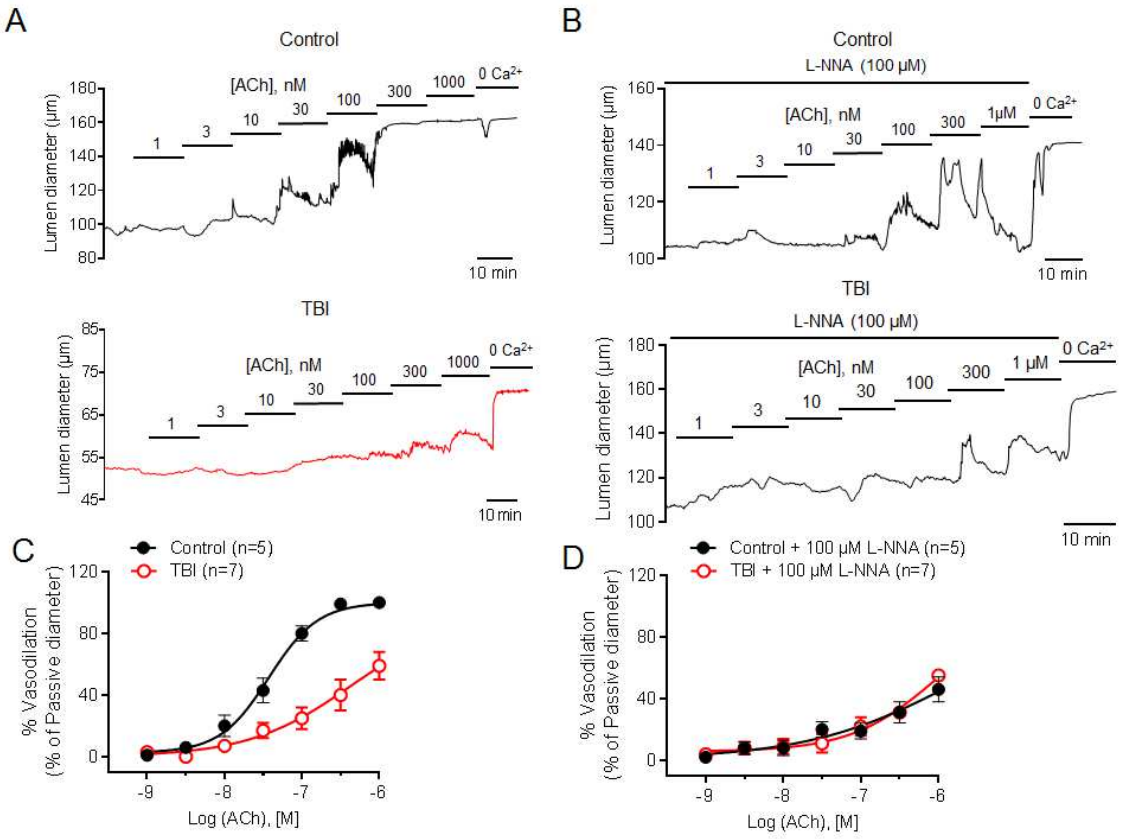

Figure 2: Endothelial dysfunction was assessed in arteries from TBI rats by an endothelial dependent agonist ACh. Representative trace of arteries from control and TBI animals (A) ; arteries were allowed to develop myogenic tone at 80 mmHg, and were then subjected to stepwise increases of the muscarinic receptor agonist acetylcholine (ACh). (B) ACh concentration curves were performed in the presence of NOS inhibitor L-NNA (100 mM) dilations. (C) Summary data of ACh response curve in control and TBI groups. (D) Summary data of ACh response curve in the presence of L-NNA in control and TBI. (Two-way ANOVA, $P<0.0001$).

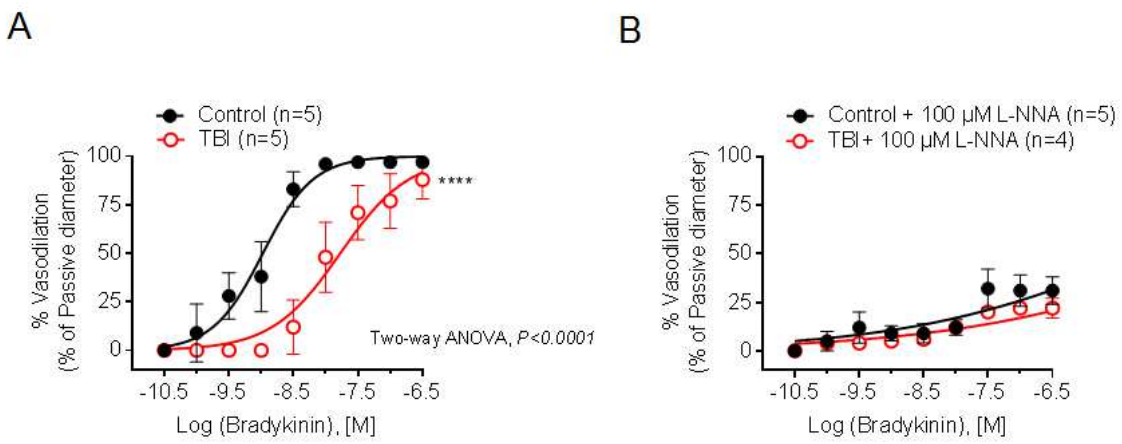

Figure 3: Bradykinin induced vasodilations are attenuated in mesenteric arteries following TBI. Concentration response curves were performed in pressurized arteries from control and TBI rats in a pressure myograph system (A)(Two way ANOVA p<0.0001). (B) Response increased concentrations of bradykinin in the presence of the NOS inhibitor L-NNA. Vasodilation (% vasodilation) was quantified as the measured vessel diameter normalized to maximal relaxation of the same artery in Ca^{2+}-free buffer

2.3.3 NO contribution to ACh-induced vasodilation following TBI

To further investigate the roles of endothelial dependent vasodilations in control and TBI arteries, the EDH pathway was inhibited with the pharmacological blockers: apamine (300 nM), charybdotoxin (200 nM), and paxilline (1 µM) (Figure 4A-B). ACh-induced dilation (1 µM) in control arteries (Figure 3A) were reduced, but not blocked (17 ± 3% of passive diameter, n=10), implying that in control groups the NO pathway is intact (Figure 3A). However, the dilation to ACh (1 µM) in the presence of these blockers was partially inhibited in TBI arteries (9 ± 3% of passive diameter, n=8, Mann-Whitney test, $P<0.05$) compared to controls (Figure 4C). The effect of SK_{Ca}, IK_{Ca} and BK_{Ca} channel blockade on basal tone was also tested in TBI and control arteries. We found no difference between groups (Figure 4D). Overall this indicates that in the absence of EDHF component, the NO associated pathway is unable to dilate arteries following a TBI.

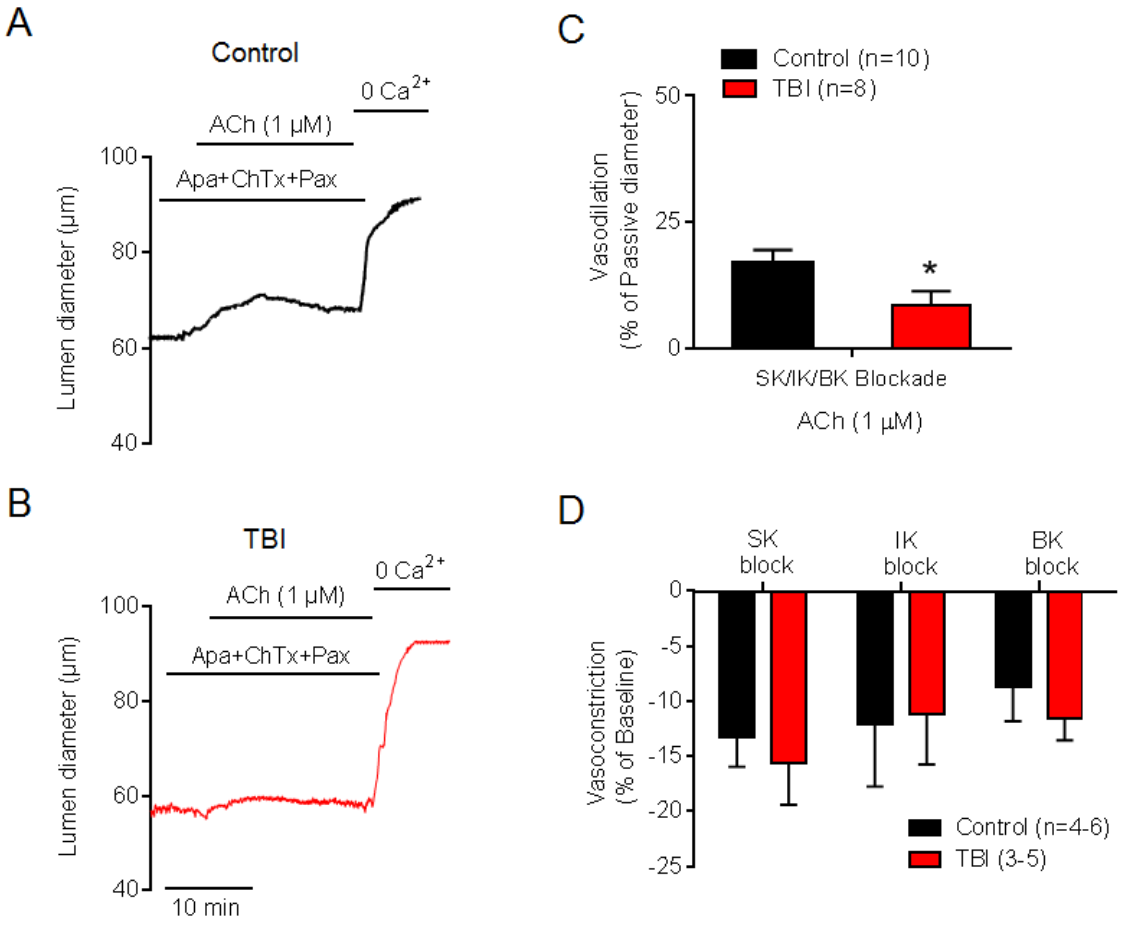

Figure 4: Endothelial dysfunction following TBI involves impairment in NO-component of vasodilation: Representative trace showing control (A) and TBI (B) arteries in the presence of pharmacological blockers of EDHF vasodilatory pathways. Arteries were incubated in the presence of apamin (300 nM), charybdotoxin (200 nM) and paxilline (1 µM) prior to addition of ACh (1 µM). Vasodilation (% vasodilation) was quantified as the measured vessel diameter normalized to maximal relaxation of the same artery in Ca^{2+}-free buffer. (C) Summary data show that TBI arteries displayed a significant diminution in the NO component of ACh-induced dilation compared to controls (Mann-Whitney test, $P < 0.05$). (D) Summary of basal activity of EDHF related channels are not different between groups.

2.3.4 NO release or production is decreased in TBI mesenteric arteries

The basal production of NO in mesenteric arteries was measured indirectly by measuring the constriction induced by the NOS competitive inhibitor L-NNA (100 μM) (Figure 4). L-NNA induced vasoconstriction was attenuated in arteries from TBI rats (3 ± 2% change in baseline, n=9) compared to controls (8 ± 1% change in baseline, n=12) (Mann-Whitney test, $P<0.05$) (Figure 5C), indicating a decreased basal NO production in TBI arteries.

Pressure-induced constriction (myogenic tone) was also measured in arteries from control and TBI animals (Figure 5D). Arteries from TBI rats showed enhanced myogenic tone compared to controls at 80 mmHg, respectively (40 ± 9 % tone, n=6 *vs* 27 ± 2% tone, n=10). Denuded mesenteric arteries from control rats displayed an enhanced myogenic tone similar to arteries from TBI rats (42 ± 7% tone, n=6 *vs* 39 ± 3% tone, n=5, n.s.) indicating that in this vascular bed, a tonic release of NO counterbalances the myogenic response. Endothelium denudation had no effect on myogenic tone development within the TBI group. Inhibition of NO production with L-NNA (100 μM) normalized the level of tone between groups (33 ± 3% tone, n=17 *vs* 35 ± 2%, n=12, n.s.) (Figure 5D).

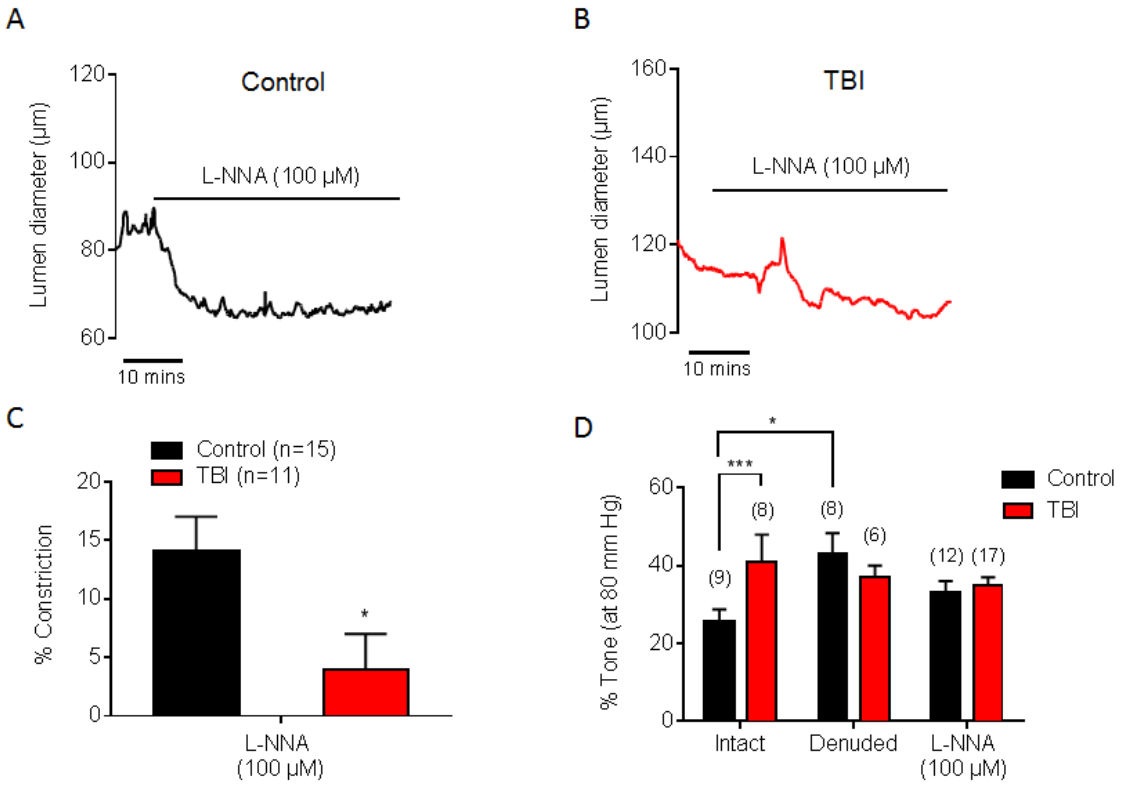

Figure 5: Decreased NO bioavailability and increased myogenic tone in TBI arteries: Representative trace of control (A) and TBI (B) arteries exposed to the nitric oxide synthase inhibitor L-NNA (100 nM), the resulting vasoconstriction was quantified as the luminal diameter normalized to the baseline diameter of the same vessel (% change in baseline). (C) Summary data of % constriction in response to L-NNA in control and TBI arteries (T-test, $P<0.05$). (D) Summary data of myogenic tone in TBI and control arteries in intact and denuded vessels, and in the presence of L-NNA.

2.3.5 Effect of L-Sepiapterin supplementation on ACh-induced dilation

Previous studies have reported that deficient NO production in vascular disease has been linked to eNOS uncoupling by oxidation of the NOS cofactor BH_4 by ROS. To test whether the lack of cofactor contributes to a decreased eNOS activity and uncoupling of eNOS, we added an exogenous substrate for BH_4 synthesis, L-sepiapterin (1 μM)[89]. Concentration-response curves of ACh (1 nM to 1 μM) were performed in control and TBI vessels in the presence of L-sepiapterin (1μM) (Figure 6). L-sepiapterin supplementation rescued the maximal dilation to ACh (1 μM) in TBI arteries (91 ± 1% of passive diameter, n=5 vs 86 ± 6% of passive diameter n=5) (Figure 5B). However, L-sepiapterin was not able to rescue the entire ACh concentration response curve in TBI. L-sepiapterin (1 μM) incubation also had an effect on ACh induced vasodilations in control vessels, attenuating ACh induced vasodilations from 30nm-1 μM (Figure 6A), suggesting that addition of BH_4 into control arteries disrupts normal vasodilatory function. The effect of sepiapterin on myogenic tone on TBI arteries was tested. We found no difference in tone between TBI and control vessels (32 ± 1% of tone *vs* 32 ± 2% of tone) (Figure 6C). Sepiapterin was able to restore arterial tone in arteries from TBI animals compared to controls. Overall, Sepiapterin partially restored ACh vasodilations (E_{max}) and fully restored myogenic tone in arteries obtained from TBI animals.

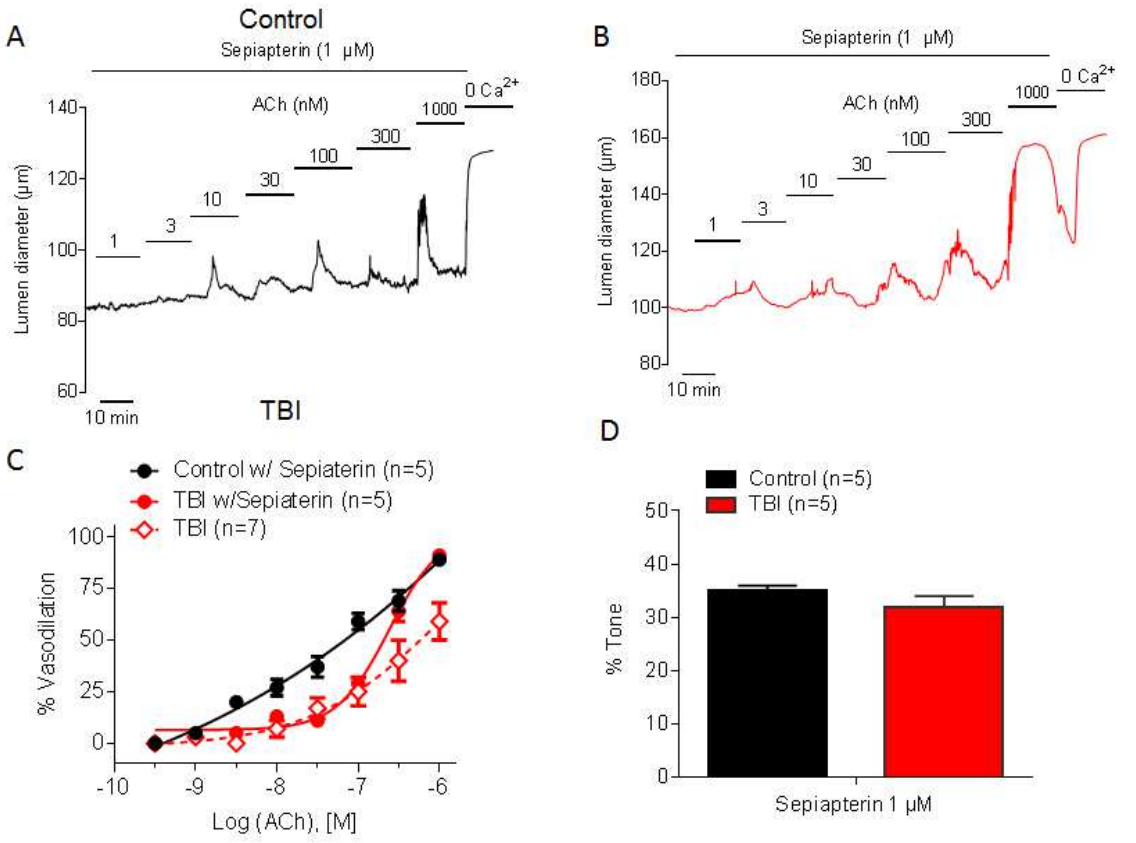

Figure 6: The BH_4 precursor L-sepiapterin partially restored endothelial dependent dilations in TBI arteries: Sepiapterin (1 µM) was incubated in control (A) and TBI (B) vessels before and during ACh response curves at 80mmHg. (C) Summary data of ACh CRC in the presence of sepiapterin. (D) Measurement of the effect of sepiapterin on myogenic tone in Control and TBI arteries. % Tone was quantified as the measured vessel diameter normalized to maximal relaxation of the same artery in Ca^{2+}-free buffer.

2.3.6 L-Arginine supplementation and arginase inhibition rescues ACh-induced vasodilations in arteries from TBI rats

The partial rescue of BH_4 synthesis through exogenous supplementation of the substrate sepiapterin lead us to change our focus from the cofactor and look at the eNOS substrate L-arginine. Control and TBI arteries were incubated with L-arginine (100 μM) before and during an ACh concentration response curve (1 nm to 1 μM). Supplementation with L-arginine rescued ACh-induced dilations in TBI arteries compared to TBI arteries without supplementation (1 μM ACh; 98 ±1% of passive vs 59 ±9% of passive) (Figure 7D).

Previous studies have shown that the enzyme arginase competes with eNOS for L-arginine and therefore may contribute to endothelial dysfunction [8,38,47,101,115]. Therefore, we tested ACh-induced dilations in the presence of the arginase inhibitor nor-NOHA (20 μM) in control (Figure 7A) and TBI (Figure 7B) arteries. Incubation with nor-NOHA rescued ACh-induced in TBI compared to TBI arteries without rescue (1 μM ACh; 98 ±1% of passive vs 59 ±9% of passive). These data indicate that increases of arginase activity and associated reduction in cellular L-arginine levels in the vascular endothelium reduces NO levels because of reduced eNOS substrate (L-arginine) availability.

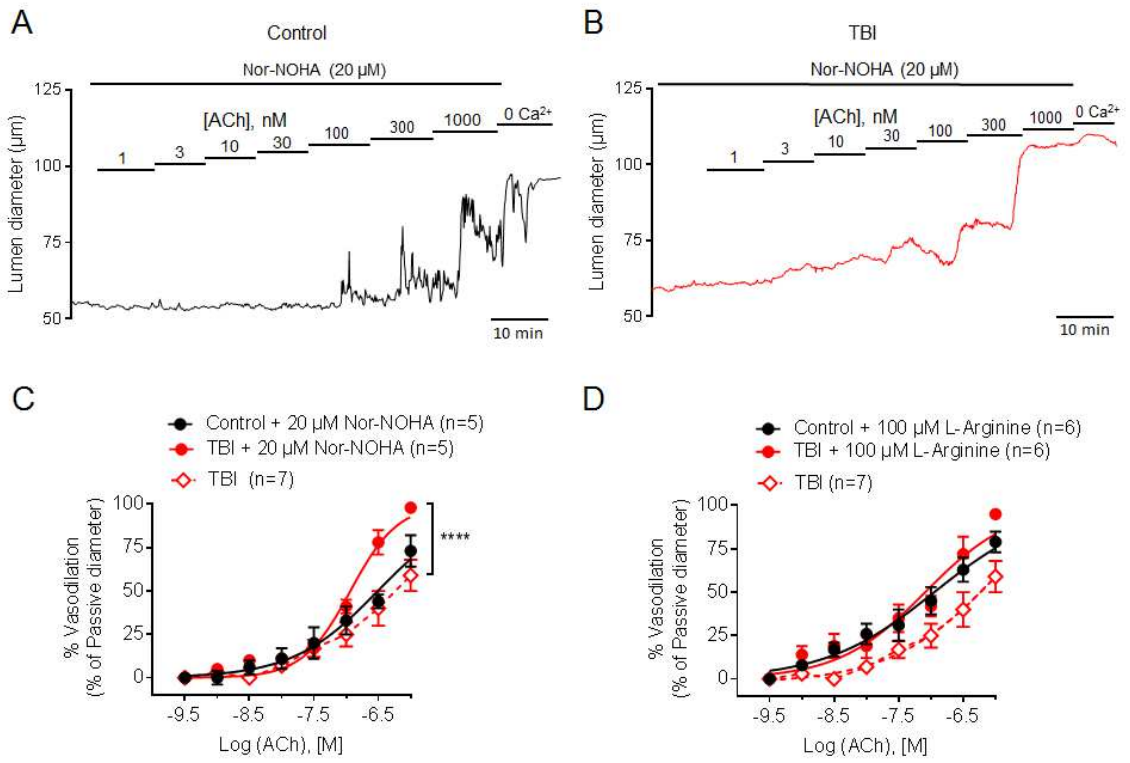

Figure 7: Supplementation of L-arginine and inhibition of arginase restores endothelial function in mesenteric arteries following TBI: ACh induced concentration response curve in the presence of the specific inhibitor of arginase Nor-NOHA (20 µM) (Nω-hydroxy-nor-Arginine) in control (A) and TBI (B) arteries. (C) Summary data showing ACh CRC in the presence of Nor-NOHA, (D) Control and Mesenteric arteries subjected to ACh CRC in the presence of L-arginine. Dotted lines represent control arteries previously shown in Figure 1.

2.3.7 Vascular smooth muscle sensitivity to NO and contractility

To determine if the vascular smooth muscle sensitivity to NO was altered following TBI, VSMC sensitivity was tested by performing a concentration-response curve with the NO-donor spermine-NONOate (1 nM to 1 μM) in the presence of L-NNA (100 μM) to prevent endogenous NO production. Spermine NONOate elicited endothelial-independent vasodilations which were not significantly different between groups (n=5-6, Two-way ANOVA, $p > .05$) (Figure 8A).

To investigate if VSMC constriction responses were changed in TBI mesenteric arteries, contractility was tested by performing concentration response curves with the agonist U-46619 (3 nM to 1 μM) (Figure 8B) at low pressures to avoid the contribution of myogenic tone (40 mm Hg). U-46619 elicited VSMC constrictions were were similar between groups (n=4, Two-way ANOVA, $p > .05$)

An alternate hypothesis for impaired NO production would be decreased eNOS expression. To further investigate whether TBI affects eNOS expression, eNOS mRNA levels were measured in isolated fourth order mesenteric arteries from control and TBI rats. Quantitative-PCR (qPCR) showed that there was no difference of eNOS mRNA levels between groups (Figure 8C).

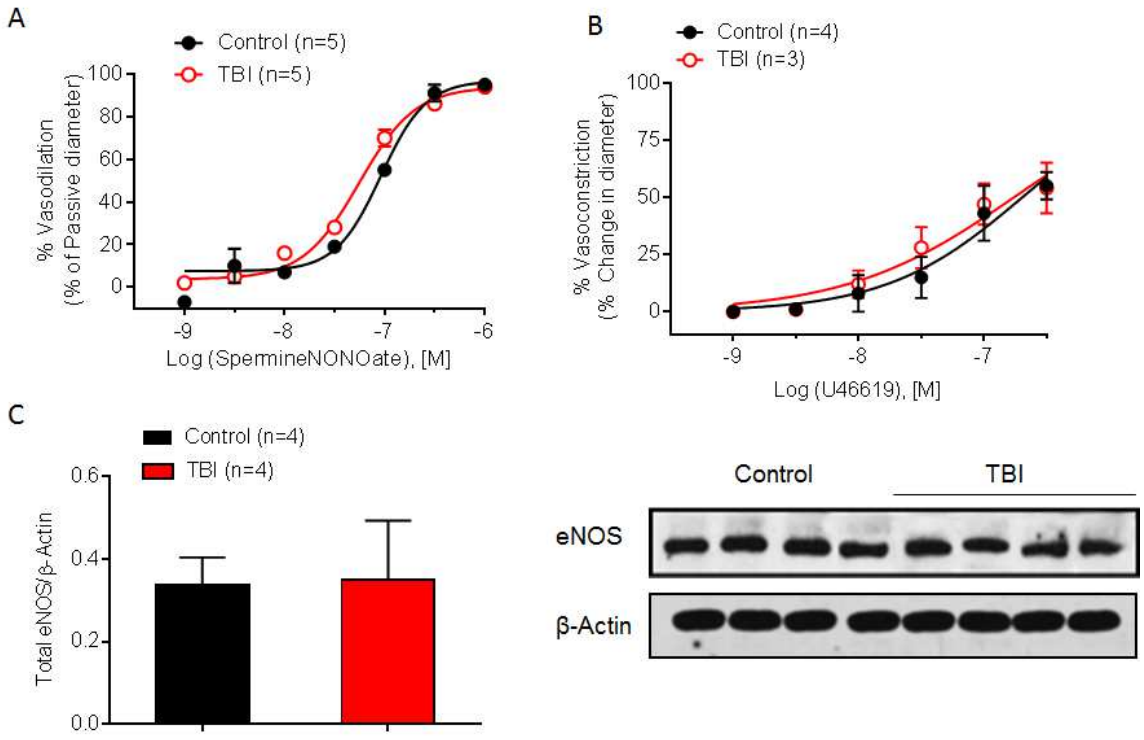

Figure 8: Smooth muscle sensitivity to NO and functional contractility is not altered in TBI vessels: (A) Smooth muscle sensitivity to NO was determined using the NO donor Sperimine NONOate in the presence of L-NNA in control and TBI arteries. (B) Smooth muscle contractility was determined by performing U-46619 CRC in control and TBI arteries. (C) mRNA expression was measured in Control and TBI arteries, there was no significant difference.

2.4 Discussion

This is the first study conducted to address the effects of TBI on areas remote from the site of injury. This study begins to address how TBI has a peripheral effect on vasculature in addition to the well-documented cerebral effect. We hypothesized that TBI causes profound changes in mesenteric arteries resulting in a loss of endothelial-dependent vasodilation 24 hours after brain injury. We were able to demonstrate endothelial dysfunction in TBI mesenteric arteries by performing concentration response curves for two different endothelial-dependent vasodilators, the muscarinic agonist ACh and BK. We found that endothelial-dependent dilations to both agonists were impaired in fourth-order mesenteric arteries 24 hours after TBI and that the NO pathway is impaired following TBI; however the EDHF pathway remained intact, providing the predominant endothelium-dependent vasodilatory mechanism in arteries obtained from TBI animals. We also studied whether eNOS uncoupling could be responsible for the lack of eNOS-mediated dilations following TBI which has been shown in other animal models of diabetes [100], hypertension [69] and obesity [116]. Supplementation with the BH_4 precursor sepiapterin partially rescued ACh-induced vasodilations (1 μM) and restored myogenic tone in arteries from TBI rats. Furthermore, we found that arginase inhibition with nor-NOHA and supplementation with L-Arginine rescued ACh-induced vasodilations in mesenteric arteries following TBI.

Our functional assay of ACh-induced dilations demonstrated that endothelial-dependent vasodilations were impaired in fourth-order mesenteric arteries 24 hours after TBI (Figure 2). When the contribution of NO was abolished by L-NNA, ACh-induced vasodilations were partially inhibited in control mesenteric arteries, indicating that ACh-induced vasodilation involves both NO and EDHF pathways (Figure 6). However, NOS inhibition had no effect in TBI arteries, suggesting the lack of NO bioavailability and contribution to ACh-induced dilations following TBI. The remaining dilation to ACh in the presence of L-NNA (100 μM) shows the EDHF component of ACh-induced dilations, which implicates the contribution of K_{Ca} channels (SK and IK) also present in the vascular endothelium which are activated by a rise in intracellular cytosolic Ca^{2+} upon agonist stimulation [108]. As Figure 1 shows, ACh-induced dilations measured in TBI rats mimicked those of control in the presence of L-NNA (100 μM) suggesting that the EDHF component is the only vasodilatory pathway that remains intact following TBI in this vascular bed. Vasoconstriction in response to apamine, charybdotoxin, and paxilline in control and TBI arteries was similar, further demonstrating that the EDHF pathway is intact following TBI (Figure 4). From these data we can conclude that the EDHF pathway is intact in mesenteric arteries following TBI.

In control arteries, EDHF represents ~50% of the maximal ACh-induced endothelial-dependent vasodilation (Figure 2C). This finding contradicts previous studies that suggest small resistance arteries rely mostly on EDHF (~75%) for vasodilation [28,54,68,97]; these differences in EDHF contribution could be due to different species or to the arterial diameter elected for study. In other models of disease such as diabetes,

disruptions of the EDHF pathway have been reported [72,106,112]. In this regard, our data do not support the findings from Leo et al, where they showed that both the NO- and EDHF-mediated relaxations were significantly diminished in mesenteric arteries from streptozotocin-induced diabetes animal model [72]. This can be attributed to the difference in disease model (diabetic vs TBI) or possibly chronicity of the disease pathway (TBI acute onset and diabetes long term). However, a study conducted on mesenteric arteries in an ischemia-reperfusion model, where they showed NOS inhibition attenuated ACh-response by ~50%, does support our findings that the NO-component contributes more to ACh-induced dilations in rat mesenteric arteries than previously mentioned [77].

We also used a different agonist to explore whether or not the endothelial dysfunction seen after TBI is unique to the muscarinic receptor ACh. Bradykinin (BK), an agonist of the B2 receptor which is also located in the vascular endothelium, induced vasodilation in control and mesenteric arteries (Figure 3). BK-induced vasodilations were significantly attenuated in TBI arteries without altering E_{max} (Figure 3). This may be indicative of a different mechanism of endothelial-dependent vasodilation in response to bradykinin or that the ACh muscarinic receptor is dysfunctional in TBI mesenteric arteries. A study done in rat mesenteric arteries *in vivo* showed that the NOS inhibitor L-NAME plus indomethacin did not inhibit BK-induced blood flow [87], implying that BK dilates vessels mainly through the EDHF pathway. Contradicting this, previous studies have proven that BK-induced dilation in mesenteric arteries functions through both the NO and EDHF pathway and can be attenuated by the NOS inhibition [68]. However, the inability

of vasculature to dilate in the presence of the agonist ACh or BK has been a reference for the loss of vascular integrity specifically for endothelial-dependent dysfunction.

When the contribution of EDHF was inhibited with apamin, charybdotoxin and paxilline, relaxations to ACh (1 μM) were significantly decreased in TBI compared to normal arteries, further suggesting that the contribution of NO was impaired by brain injury (Figure 3). To measure basal NO production, the constriction to L-NNA was measured in arteries from control and TBI animals. Control arteries expectedly constricted ~20% (compared to tone) in response to L-NNA which is consistent with previous studies [61,72,81]. However, constriction to L-NNA in arteries taken from TBI animals was significantly decreased compared to controls (Figure 6C) indicating that basal level of NO production is decreased in mesenteric arteries from TBI rats demonstrating an impaired NO pathway in systemic circulation following TBI. In comparison, in cerebral arteries NO production is increased following TBI compared to controls [111]. This might be a compensatory mechanism of traumatic brain injury found centrally and not peripherally.

Vascular tone at 80 mm Hg was also measured in mesenteric arteries from control and TBI groups. TBI arteries displayed an enhanced myogenic tone compared to controls (Figure 4D). Denuded TBI arteries had no difference in myogenic tone compared to intact TBI, however, control denuded arteries displayed an enhanced myogenic tone similar to that of TBI arteries. Inhibition of NOS with L-NNA in control arteries diminished the NO component and was similar to the myogenic response of TBI arteries, demonstrating that increased myogenic tone in TBI rats is due to an impaired function of eNOS. Although the role of endothelial vasoactive factors in modulating myogenic tone has been

controversial, in this study we have correlated the lack of NO production with an increase of myogenic tone. This increase in basal constriction was not due to altered sensitivity to NO (Figure 7A) or to increase of VSMC contractility (Figure 7B) since both remained intact in TBI mesenteric arteries.

After confirming endothelial dysfunction in TBI mesenteric arteries, we studied whether a decreased concentration of the essential eNOS cofactor BH_4 promoted eNOS uncoupling, resulting in a lack of NO-derived vasodilations in response to ACh. Supplementation with sepiapterin, an intermediate in the salvage pathway of BH_4 synthesis, showed partial rescue to ACh-induced vasodilation at the maximal concentration of 1 μM (Figure 6C) and restored myogenic basal tone compared to controls (Figure 6D) but was unable to rescue concentrations of 1 nM-300nM of ACh. Sepiapterin rescue at only 1 μM ACh could imply that the muscarinic receptor sensitivity to ACh is impaired in mesenteric arteries following TBI. Sepiapterin rescue of maximal dilation is consistent with the obesity model of vascular disease, where 3rd order mesenteric arteries were rescued from attenuated vasodilations to ACh at 1 μM [89]. However, a concentration response curve was not performed in this study so we were unable to compare our results obtained at lower concentrations of ACh.

Recent data suggest that the ratio of BH_4:BH_2 may be a more important determinant of eNOS function than the absolute concentration of BH_4 [56,109,110]. In an oxidative environment BH_4 can be oxidized to BH_2 which has a higher binding affinity to eNOS than BH_4. Although BH_4 oxidation was not investigated in this study, increased

concentrations of BH_2 may explain why supplementation with sepiapterin was not able to rescue eNOS function in TBI arteries. On the other hand, high concentrations of sepiapterin could also uncouple eNOS leading to generation of superoxide O^{2-}. Recently, it has been shown that BH_2 and sepiapterin inhibit NOS in vitro by displacing the pre-bound BH_4 with >80% efficiency [58]. BH_2 and sepiapterin are metabolites that accumulate in patients with variants of BH_4 deficiency, and it has been suggested that they may potentiate the superoxide formation during the uncoupled reaction of NOS [9,119].

The BH_4 precursor sepiapterin was unable to rescue the concentration-response curve of ACh which lead us to investigate whether the eNOS substrate, L-arginine, could explain compromised ACh-induced vasodilation following TBI. We discovered that exogenous supplementation with the NOS substrate L-arginine (100 μM) did rescue ACh-induced vasodilations in TBI arteries (Figure 7D). Since arginase and eNOS share the same substrate, an increased arginase activity or expression could decrease L-arginine bioavailability. In this regard, several studies have shown the role of arginase in endothelial dysfunction and eNOS uncoupling under different physiological and pathological states [8,44,47,57,90,101]. Incubation with the specific inhibitor of arginase nor-NOHA (20 μM) rescued ACh-induced dilations in our model of TBI (Figure 7A-C). Further studies measuring total arginase expression using western blot analysis will be necessary to determine if increased arginase is responsible for uncoupling eNOS and promoting endothelial dysfunction following TBI. Despite the selectivity of arginase inhibitors, it has been reported that nor-NOHA can potentially inhibit eNOS in mesenteric

arteries; supplementation of L-arginine + nor-NOHA however rescued ACh-induced vasodilation in control mesenteric arteries [53].

In our study, we did not find increased eNOS expression in mesenteric arteries following TBI (Figure 8). Similar findings were seen in an ischemia reperfusion model where eNOS protein level expression measured in mesenteric arteries was also not increased. Also, in an obesity model, eNOS expression was not different between control and disease groups, however, eNOS activation measured by phosphorylation was decreased, contributing to impaired endothelial-dependent dilations [116]. In mesenteric arteries from a rat model of obesity it was reported that there was no difference in total eNOS expression; however, eNOS dimerization suggested that eNOS was acting in a monomeric state which was responsible for uncoupled behavior [72]. Although we determined that eNOS expression is not altered following TBI, it is also possible that the activity of eNOS is altered by post-translational modification or uncoupling and un-dimerization.

Finally, because there has been some controversy about the role of endothelial vasoactive factors in regulating myogenic tone, we also tested the vascular smooth muscle function to rule out VSMC dysfunction as mechanisms of impaired vasodilation (Figure 7). We tested both vasodilation in response to NO and contractility in response to the thromboxane agonist U-46619, and found no differences in VSMC function at 24 hours after TBI compared to controls. Thus, the increase in vascular tone after TBI is not due to change in sensitivity to NO (Figure 8A) or altered contractility of VSMC (Figure 8B) but due to the dysfunctional endothelium.

The vascular effect seen remote from the site of injury might suggest the contribution of circulating factors such as histones, polyphosphates or other unidentified factors. Recently, they have been implicated in TBI [66] and other pathological states like sepsis [114] or lung-associated injury [1]. Whether they cause endothelial damage to resistance-sized arteries remains unclear. In summary, this study demonstrates that fluid percussion induce TBI causes profound vascular systemic damage to mesenteric arteries, leading to a dysfunctional TBI induces a release of circulating factors into the systemic circulation, causing a dysfunctional endothelial-dependent vasodilation in mesenteric arteries and increased arginase activity (Figure 9). Arginase in turn competes with eNOS for L-arginine; consumption of L-arginine by arginase starves eNOS causing a loss of eNOS function. This in turn diminishes NO-production in the vascular endothelium resulting in a compromised endothelial-dependent vasodilation to agonist stimulation (ACh and BK) and increased myogenic tone due to a decreased basal NO production.

2.5 Conclusions and Future Directions

Traumatic injury to brain tissue causes release of circulating factors normally sequestered from the circulation, along with massive catecholamine release and elevations in ROS. Several studies have shown that TBI can lead to cardiovascular pathology, but to our knowledge, there are no studies focusing specifically on the vascular endothelium after TBI. This is an important area for research, because endothelial dysfunction could explain the hypertension, coagulopathy and impaired barrier functions of pulmonary and cerebral vessels, often seen after trauma.

Overall, this study demonstrates 24 hours after a traumatic brain injury, systemic resistance vessels show signs of endothelial dysfunction, manifest as impaired endothelial-dependent vasodilation. This appears to be due to an increased activity or expression of arginase activity in the endothelium. This increase in arginase activity or expression subsequently leads to competition for L-arginine, limiting the ability of eNOS to produce of NO (Figure 9). These results have important clinical implications. Resistance sized arteries which are extremely important in regulating blood pressure, circulating blood volume, and the production of anti-coagulant factors. This damage to vascular endothelium could contribute to the dysfunctional coagulation factors seen in patients after trauma. Thus, our data suggests that arginase inhibition may be a potential therapeutic target to rescue endothelial function in TBI patients.

Future studies can be performed to determine if the activity of eNOS is altered by post-translational modification through Western blot analysis can determine if eNOS is acting in a coupled or uncoupled form, or if its active form (phosphorylated) is being expressed. Also, it is unknown if the arginase enzyme has an increased protein expression in the endothelium or if its activity is increased by post-translational modification. This can be later determined with western blot analysis of protein levels and phosphorylization of arginase. The isoform of arginase can be determined by immunohistochemistry of fixed sections of endothelium. Further studies using the slit open artery preparation (Figure 10) incubated with the fluorescent indicator DAF-2 DA of 4th order mesenteric arteries can help distinguish if the production of NO is altered following TBI. To test if there is an increased production of ROS in the mesenteric

vascular endothelium, further studies using immunohistochemistry in control and TBI arteries could be performed.

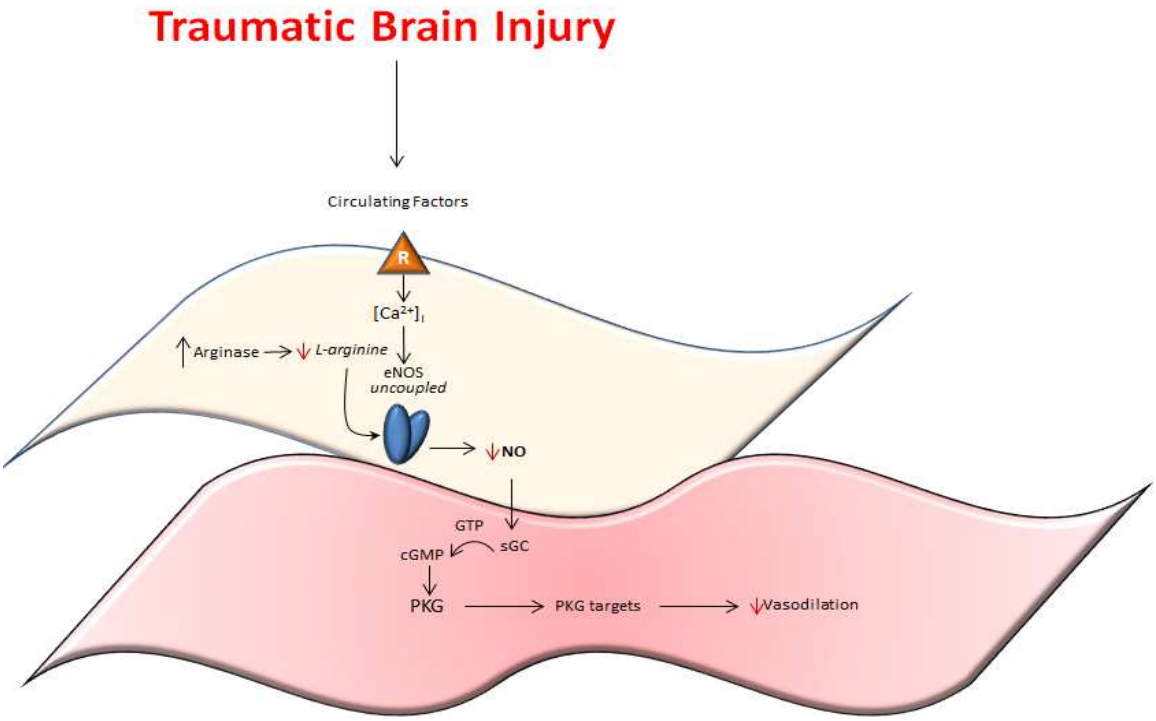

Figure 9: Schematic for endothelial dysfunction following TBI: Traumatic Brain Injury causes a release of circulating factors into the systemic system causing an increase of arginase activity or expression in vascular endothelium. Increases of arginase introduces a competitive environment for L-arginine, starving eNOS for L-arginine and causing eNOS uncoupling. eNOS can no longer produce NO, this leads to a decrease of basal and agonist induced NO production and an increase of myogenic tone.

2.6 Limitations

We acknowledge the limitations of this study. One possible limitation is the 24 hour time point of this study. We recognize that systemic pathophysiological outcomes of TBI can last for up to two weeks after injury. However, we chose one time point to ensure consistency of injury and outcome. Second, only male Sprague Dawley rats were investigated in this study and the findings cannot be generalized to the female rat population due to the neuroprotective mechanisms in the female rat [2,27]. Third, we did not assess the prostacyclin pathway of endothelial-dependent dilations due to various accounts that have shown that the addition of the cyclooxygenase (COX) inhibitors has no effect on ACh-induced vasodilations in control arteries [89,97,112]. Specifically in rat mesenteric arteries, the contribution of the prostacyclin pathway in endothelial mediated vasodilation is controversial. However, other models of disease have shown a tendency of an increased cyclooxygenase derived products such as PGI_2 when there is an inhibited or impaired NO contribution [68,72]. Fourth, we did not explore the levels of ROS because it was beyond the scope of this project. It is apparent that ROS may play a role in mesenteric artery dilation following TBI. ROS have been a common denominator when studying endothelial dysfunction in vascular disease and is a hallmark of endothelial dysfunction and eNOS uncoupling.

CHAPTER 3: SUPPLEMENTARY METHODS AND RESULTS

3.1 Slit open preparations

To assess basal production of NO in control animals, mesenteric arteries were dissected from surrounding tissue and were pinned down and slit open on a sylgard plate with the endothelium facing upwards (Figure 10) [111]. The artery was incubated with the NO fluorescent indicator assay 4.5-diaminofluorescein (DAF-2 DA; 10 μM) for 1 hr at 32°C in the dark and was then imaged with a spinning disk confocal microscope at 30 frames/sec as previously described [111]. The vessel was superfused with oxygenated PSS and warmed to 37°C for 15 minutes before recordings to allow deesterfication of the fluorescent indicator. Fluorescence intensity and pseudocolor images were analyzed with a custom software (Sparkan, Dr. Bonev, University of Vermont). Unfortunately an increase of NO production in the presence of the muscarinic receptor ACh was not easily detected in control arteries. We speculated that the reason for this may be that NO release in mesenteric arteries due to ACh was too small to detect with the DAF-2 DA which has a detection limit of 2-5 nM. The viability of the preparation may have been compromised during the slitting open and pinning; over stretching the vessel may have damaged or torn endothelial cells, making imaging extremely difficult.

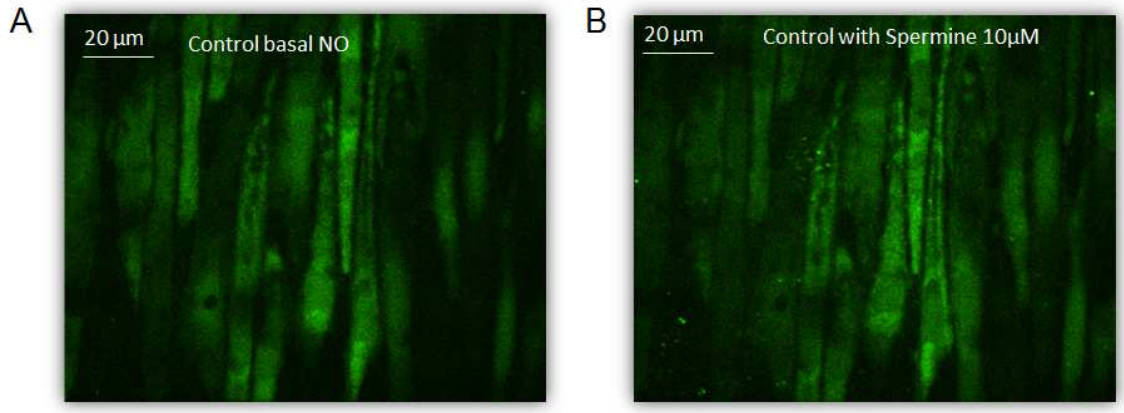

Figure 10: NO production in the endothelium from slit open arteries assessed by DAF-2 fluorescence: en face preparation of 3rd order control mesenteric arteries showing NO staining indexed by DAF-2. Addition of NO donor Spermine (10 µM) slightly increased the NO production in endothelium.

3.2 Cryostat sections

Fourth order mesenteric arteries were dissected and were briefly cannulated to remove intraluminal blood before freezing. Arteries were placed in OCT freezing medium and then frozen in 2-methyl-butane chilled with dry ice for 30 sec. Frozen sections (100 uM) were made with a cryostat; the sections were then incubated with 10 µM DAF-2 DA for 30 minutes at room temperature (Figure 11). Negative controls were not incubated with DAF-DA, control and TBI arteries were incubated with DAF-2 DA and were imaged, control and TBI arteries were challenged with ACh (100 µM) and imaged. These data were not incorporated due to the difficulty of viewing the endothelium in the arterial cross sections. Although overall NO in the artery seems to be elevated in the controls compared to TBI, we decided not to continue with this technique due to the lack of proper characterization of NO (DAF-2 DA) staining in the endothelial layer.

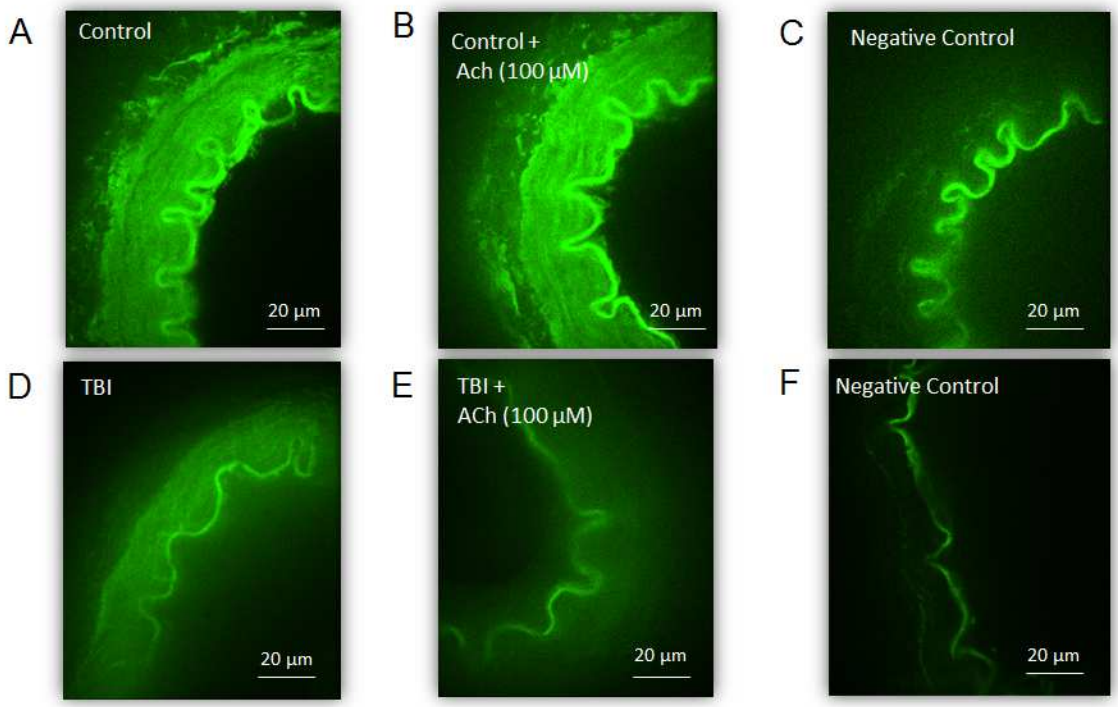

Figure 11: NO production in fresh (unfixed) frozen sections of mesenteric arteries: NO production indexed by the fluorescent indicator DAF-2 DA in 100 µm sections of mesenteric arteries in control (A) and TBI (D) arteries (n=1). Application of ACh (100 µM) on both groups (B & E), Negative Controls showed autofluorescence in the absence of DAF-2 DA (C & F)

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