2012

BLAST-INDUCED BRAIN INJURY: INFLUENCE OF SHOCKWAVE COMPONENTS

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BLAST-INDUCED BRAIN INJURY: INFLUENCE OF SHOCKWAVE COMPONENTS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Lexington, Kentucky

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Lexington, Kentucky
2012

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ABSTRACT OF DISSERTATION

BLAST-INDUCED TRAUMATIC BRAIN INJURY: INFLUENCE OF SHOCKWAVE COMPONENTS

Blast-induced traumatic brain injury (bTBI) has been described as the defining injury of Operations Enduring Freedom and Iraqi Freedom (OEF/OIF). Previously, most blast injury research has focused on the effects of blast on internal, gas filled organs due to their increased susceptibility. However, due to a change in enemy tactics combined with better armor and front-line medical care, bTBI has become one of the most common injuries due to blast. Though there has been a significant amount of research characterizing the brain injury produced by blast, a sound understanding of the contribution of each component of the shockwave to the injury is needed. Large animal models of bTBI utilize chemical explosives as their shockwave source while small animal models predominantly utilize compressed air-driven membrane rupture as their shockwave source. We designed and built a multi-mode shock tube capable of utilizing compressed gas (air or helium)-driven membrane rupture or chemical explosives (oxyhydrogen – a 2:1 mixture of hydrogen and oxygen gasses, or RDX – high order explosive) to produce a shockwave. Analysis of the shockwaves produced by each mode of the McMillan Blast Device (MBD) revealed that compressed air-driven shockwaves exhibited longer duration positive phases than compressed helium-, oxyhydrogen-, or RDX-driven shockwaves of similar peak overpressure. The longer duration of compressed air-driven shockwaves results in greater energy being imparted on a test subject than would be imparted by shockwaves of identical peak overpressures from the other sources. Animals exposed to compressed air-driven shockwaves exhibited more extensive brain surface hematoma, more blood-brain barrier compromise, more extensive reactive astrocytosis, and greater numbers of activated microglia in their brains than did animals exposed to oxyhydrogen-driven shockwaves of even greater peak overpressure. Taken together, these data suggest that compressed air-driven shockwaves contain more energy than their chemical explosive-derived counterparts of equal peak overpressure and
can result in greater injury in an experimental animal model. Additionally, these data suggest that exposure to longer duration shockwaves, which is common in certain real-world scenarios, can result in more severe bTBI. The results of this study can be used to aid design of blast wave mitigation technology and future clinical intervention.

KEYWORDS: Improvised Explosive Device, Military, Blast Injury, Brain Injury, Secondary Pathology
BLAST INDUCED BRAIN INJURY:
INFLUENCE OF SHOCKWAVE COMPONENTS

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2 August 2012
For my parents, grandparents and wife who have provided unwavering support throughout life’s journeys, this journey, and those to come.
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all my teachers throughout my education that have been instrumental in the formation of my ideas and academic abilities. I would like to thank the members of my dissertation advisory committee Drs. Edward Hall, Jonathan Lifshitz, Joe Springer, and Kathryn Saatman for their helpful discussion, guidance, and mentorship throughout the years of preparation represented in this work. I must also thank my mentor, Dr. James Geddes, for his continued support of my education. It is under his astute guidance and mentorship that I have been able to become the scientist I am today. I also thank the members of our laboratory Drs. Aashish Joshi, Jordan Clark, RamaKrishna Badugu, Yanzhang Li, Brantley Graham, Colin Rogers, ChenGuang Yu, Sarbani Ghoshal, Ms. Vimala Bondada, Ms. Ranjana Singh, and Ms. Carolyn Crowdus, Ms. Julie Corkins and other colleagues Dr. Ryan Readnower, Mr. Shaun Carlson, and Mr. Darren Miller for their help, advice, and for always being available to lend an ear when I needed someone to listen. I also thank Mr. Rick Hisel and GLR Enterprises, LLC. for support, guidance, mechanical instruction, and friendship.

In many ways, each of us have our families to thank for the people we ultimately become. Fortunately, I have a lot for which to be thankful. I must thank my parents, Rex and Holly Reneer, for constantly challenging me to become the best I can be, for rejoicing with me when my best enabled me to accomplish my goals, and for understanding and holding me up when my best wasn’t quite good enough. To my loving wife, Dr. Mary Catherine Reneer, I am so thankful I have been able to experience this part of my life with you. Thank you for your support and love – past, present, and future.
To all of my family, you have been and continue to be a blessing; an oasis in the desert.

For those who I have neglected to mention here, please forgive me and accept my sincere thanks for your help along the way.

Lastly, I must thank God for the faith, strength, and salvation He has given to me. During these years I feel I have learned the meaning of being able to do all things through Christ, who strengthens me (Philippians 4:13).
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1. CHAPTER 1

Introduction and Background

1.1 Overview of Traumatic Brain Injury

1.1.1. Traumatic Brain Injury Epidemiology

Traumatic brain injuries (TBIs) are estimated to number approximately 1.7 million per year in the United States. Of those injuries, approximately 275,000 are nonfatal but require hospitalization, 1.1 million require treatment and release from the emergency department, and 52,000 result in death [1]. It is also estimated that up to 43% of those released from hospitalization following TBI develop long-term disabilities as a result of their injuries [2]. The yearly total number of non-reported TBIs is unknown. The financial burden of TBI is substantial due to both direct medical costs and the indirect cost of loss of productivity and totaled approximately $76.5 billion dollars in the United States in the year 2000 alone [3]. Major risk factors for TBI in the United States appear to be age, sex, and socioeconomic status [4]. Transportation-related accidents (motor vehicles, bicycles, pedestrian-vehicle accidents, and recreational vehicles) account for the majority of TBIs in the United States [5].

1.1.2. Traumatic Brain Injury Defined

Though there is no concrete consensus as to what defines a TBI, it is generally defined as damage to the brain resulting from external mechanical force [6]. Further classification of TBI can be made with respect to mechanism (closed vs. penetrating),
clinical severity (Glasgow coma scale – GCS), and by clinical evaluation of physical/structural damage (neuroimaging, surgical visualization) [6]. One advantage of using neuroimaging as a diagnostic tool is that the visualization of damaged areas is not confounded by other medical interventions such as sedation, intoxication, or paralysis [7, 8]. Nevertheless, diagnosis and classification of head injury by imaging alone is flawed due to descriptive and technological limitations combined with its temporally incremental nature [6, 9]. Alternative and complementary approaches such as classification by prognostic risk [10-13] and CSF/Serum borne biomarkers [14-17] are gaining popularity.

The injury to the brain caused by traumatic events can generally be divided into two distinct sources. Primary damage is caused directly by the application of external mechanical forces, such as impact, rapid acceleration or deceleration, a penetration, or blast waves, applied to the head [6] and cannot be prevented by pharmacologic or surgical intervention. Mechanical damage can be focal or diffuse, however it is common for both to occur in an individual who sustains a head injury [18]. Secondary damage occurs in the hours to weeks following the initial injury as a result of processes initiated by the initial mechanical trauma [19, 20]. These secondary injury mechanisms often result in further damage to the injured person. In contrast to most other forms of traumatic bodily injury, TBI patients often continue to deteriorate in the days and weeks after the event with approximately 40% of TBI patients deteriorating after hospitalization [21, 22]. Secondary injury mechanisms that can last for days or weeks after the initial insult include cerebral edema, hypoxia and hypotension, excitotoxicity, oxidative/free radical-induced stress and damage, and inflammatory processes [6]. Additionally,
cerebrovascular compromise in the form of vascular rupture or opening of the blood-brain barrier through relaxation of endothelial tight junctions can lead to further homeostatic imbalance including decreased cerebral perfusion pressure and edema [23, 24].

Astrocytosis, or infiltration of astrocytes into the area of injury, may contribute to necrotic tissue containment and/or containment of subsequently released necrotic cell contents [25] or may contain, and possibly nurture new neurons, in the months following the injury [26]. While some amount of spontaneous regeneration following brain trauma occurs [27], there are significant impediments to more robust sprouting. Chondroitin sulfate proteoglycans (CSPGs) expressed by astrocytes in the glial scar formed around areas of injury are known inhibitors of axonal growth, therefore astrocytosis could also contribute to halted axonal regrowth or healing mechanisms in the brain similar to that seen in spinal cord injury [28-35].

Trauma is known to be precipitous to activation of microglia from their resting state, thus facilitating migration to the injured regions of the brain [36-38]. Microglia serve a multifactorial role in the damaged nervous system. In addition to the classic phagocytic debris removal, they also secrete a variety of cytokines which have consequently been shown to be elevated following TBI [39]. Wallerian degeneration and microglial activation have been found to occur concurrently throughout the CNS [40, 41], effects which have been found to persist for up to 6 weeks in rats [40] and up to one year in monkey [41]. The role and function of microglia and the release of cytokines
following TBI remain controversial as their effects may be neuroprotective, neurodegenerative, or neuroregenerative [42].

1.1.3 TBI Models

Traumatic brain injury models can be divided into two general categories: direct head impact, and impact/inertial acceleration/deceleration. One of the most common models of brain injury in use today is the controlled cortical impact (CCI) model, first developed by Lighthall in 1988, which uses a mechanically controlled rigid impactor that impacts the intact dural surface of the brain [43]. A key advantage of CCI as a model is the ability to control injury parameters such as velocity, depth, and time of deformation [44-46] without the risk of rebound injury from a dropped weight [47]. Weight drop models employ a weight of pre-determined mass dropped from varying heights guided by a tube onto the dural surface [48]. The weight can also be dropped onto a surrogate metal plate fixed to the skull or onto the intact skull itself and can rebound causing additional impact events that may confound results [47]. Fluid percussion is the most common model of brain injury currently in experimental use [47]. In this model, a trephane is placed either midline or lateral to the saggital suture through which a fluid bolus is rapidly introduced into the epidural space [49-51]. Injury severity is controlled by the height of a pendulum that impacts a fluid-filled reservoir connected to the trephane through a Leur taper fitting [50]. Each of these models has been reported to produce axonal injuries, contusions, cavitation (with the exception of midline fluid percussion and
closed-head weight impact), secondary injury mechanisms including astrocytosis and microgliosis [43-46, 48-52], as well as cognitive and motor dysfunction [49, 50, 53].

Traumatic brain injury independent of direct head impact, but associated with rapid acceleration/deceleration events can be modeled using head acceleration modeling in which a pig head is affixed tightly to the injury device and the head is rotated very rapidly in the coronal plane. Reports of the injury produced by this model are extensive and are characterized by widespread axonal damage located deep in white matter tracts [54-57].

1.1.4 TBI Treatment and Injury Mitigation Strategies

Though numerous pharmacologic targets have been identified for attenuation of secondary injury mechanisms, no pharmacologic interventions have been approved for human use in the United States to-date [10]. Current treatments involve osmotherapy through the rapid infusion of mannitol to mobilize water across an intact blood-brain barrier [58] with the goal of improving focal cerebral blood flow [59]. Surgical decompression to reduce intracranial pressure (ICP) can decrease mortality, however morbidity is not always decreased [60] and there is a significant risk of severe, adverse side-effects [61]. Future surgical or pharmacologic therapy will depend on the characteristics of the individual injury, thus necessitating the need for accurate information about conditions that lead to injury and which primary and secondary injury mechanisms are relevant to a particular injury paradigm.
Apart from education and avoidance strategies, the primary tool for decreasing the incidence of primary TBI is through mitigation technologies such as seat belts, airbags, helmets and padded surfaces. With respect to brain injuries derived from physical impact, helmets are the primary method utilized to change the linear acceleration curve by lengthening the duration of the impact and decreasing the magnitude of the impact [62]. If present at all, helmet standards not only differ across the globe, but also within the US [63]. The Snell Memorial Foundation standard, while more stringent in terms of allowable total G-force transference, does not require that the G-force be spread over any certain time. Conversely, the DOT standard stipulates that the total amount of time the G-force exceeds 150 X g must not exceed 4 ms and the total amount of time the G-force exceeds 200 X g must not exceed 2 ms [63]. No helmet can completely protect from any situation and it is crucial to both better understand the physical characteristics of the forces applied to the brain during the primary injury process as well as the secondary mechanisms associated with further degeneration.
1.2 Blast Injury

1.2.1 Blast Physics

Blast occurs via the rapid conversion of an explosive solid, liquid, or gas into a rapidly expanding gas [64]. This chemical reaction releases energy in the form of a supersonic shockwave in addition to thermal, acoustic, and electromagnetic components. Rapid expansion of the blast into the surrounding atmosphere creates a near instantaneous rise in pressure to a peak pressure, termed the peak overpressure, followed by a slow, exponential decay or return to atmospheric pressure. Evacuation of atmospheric gasses caused by the expanding shockwave front can result in a negative or below atmospheric pressure-level phase. A visual representation of an ideal shockwave from a spherical source in an open-air environment can be mathematically described by the Friedlander equation (Fig. 1.1) [65]. Enclosures and distance from the explosion are two variables often experienced in the field that can change the positive phase duration of a blast wave [64, 66, 67]. Victims near or within hard surfaces often suffer from substantially greater injury [68]. In fact, very rarely is an individual exposed to an ideal blast that could be described by the Friedlander equation [69].
Fig. 1.1. The Friedlander wave. The Friedlander wave describes an ideal blast from a spherical source in an open environment. $T_0$ is the time at which the pressure began to rise above ambient pressure. Positive magnitude (Pos. Mag.) is the difference between peak pressure and ambient pressure. Positive duration (Pos. Dur.) is the time between $T_0$ and when the pressure goes below ambient pressure. Positive impulse (Pos. Impulse) is the integral of the pressure-time trace during the positive phase. Negative magnitude (Neg. Mag.) is the difference between ambient and peak negative pressure. Reprinted with permission (Appendix I) from [70].
1.2.2 Blast Injury

Blast injuries have most likely existed since man began to use explosives [67]. Early accounts of blast injury-like symptoms date to the Civil War where it was termed “Irritable Heart” [71]. Only in the 1930’s and 1940’s did blast injury research begin to receive the amount of attention it does today [67]. Blast injury research prior to the previous two decades primarily focused on the effects of blast on the lungs [67, 72, 73]. Due to the propensity of shockwaves to deposit energy where they are reflected or where their frequency changes, and areas of the body with tissue density differentials, such as the gas-filled organ-air interfaces, are most susceptible to injury [74]. Blast lung injury is characterized by pulmonary contusion, rupture of alveolar capillaries, hemorrhagic lesions, extravasation of fluid and proteins, and reduced circulation [75]. Respiratory insufficiency caused by blast-induced lung injury combined with cardiovascular irregularities like apnea, hypotension, and hypoxic pulmonary hypertension are significant factors in mortality rate following blast exposure [74]. Hemoglobin from extravasated blood and the release of vasoactive and pro-inflammatory factors leads to secondary injury cascade which can further compound the injury in a way that echoes the course of traumatic brain injury [76]. Though blast-induced lung and intestinal injury is still a serious factor in the ability of individuals to survive blast, gas-filled organ injury has significantly diminished in recent warfare due to improvements in body armor technology [77].
1.2.3 Blast-induced Traumatic Brain Injury (bTBI)

Blast-induced lung, intestine, or ear injuries have been the primary concern following blast until the recent conflicts where blast-induced traumatic brain injury (bTBI) has become the signature injury [78-81]. The increased prevalence of bTBI in recent conflicts is due to improved armor and blast-wave mitigation technologies [81-84]. Additionally, more soldiers are surviving blasts to which they would have succumbed a half a century ago thanks to reduced time to care and better medical technologies available to wounded soldiers [77]. Estimates of TBI in wounded soldiers range from 8-22%, with further estimates expecting up to 15% of infantry soldiers returning from Iraq will have sustained a TBI [80, 85]. Compared to 0.6% of the civilian population in the United States yearly experiencing TBI [1], these estimates put soldiers at high risk of sustaining a TBI [80, 85]. It should be noted that civilians are also at risk due to industrial accidents and terrorist activities [69, 82].

The mechanisms through which blast injures the brain are not known, however some leading hypotheses and experiments on how blast waves interact with tissues have shed light on possible bTBI mechanisms. Early studies suggested spallation, or the reflection of the shockwaves at the interface of mediums of two different densities, could be the source of blast-induced lung injuries [86]. Further speculation into the mechanism of blast-induced tissue damage suggested that rapidly expanding gas bubbles that had previously been compressed by the passing shockwave, could expand and damage surrounding tissue [86-88]. Additionally, attempts have been made to predict the interaction of blast waves with the skull. Computational modeling of blast waves on
helmeted individuals highlight the probability of skull flexure and discreet areas of focal stress in response to shockwave exposure as well as the importance of helmet design on focusing waves on the skull [89].

Previous studies of bTBI have largely focused on the maximal overpressure as the critical factor responsible for brain damage. However, peak overpressures resulting in CNS injury have varied widely – from 20kPa [90], to 10,000kPa [91]. Closer examination indicates a range from 20kPa to 240kPa for whole-body exposure in rats [90, 92], 150 to 396kPa for head-only exposure [93, 94], and up to 10,000 kPa for direct brain exposure [91]. This wide range of overpressures associated with bTBI suggests that additional factors may be involved. The duration of the overpressure phase and the positive impulse (integral of overpressure x duration) are known to influence the extent of blast-induced lung injury [67]. However, it is unclear if mechanisms responsible for damage to air-filled organs are similar to those associated with bTBI. The overpressure durations in previous bTBI studies vary from 0.3ms to 53ms [92, 95].

The pathophysiological characterization of blast-induced brain injury is still at a relatively early stage. There is no consensus as to the pathological consequences, however the most salient feature present in most models includes hemorrhage and blood-brain barrier breakdown [24, 94, 96, 97]. Subdural hemorrhage and cerebral contusions have been reported both in human cases [98-101] and in rats [70, 91] following blast exposure. Across injury models, vascular compromise appears to be a common marker of blast injury. Blood-brain barrier breakdown has been detected in rat models of traumatic brain injury in the lateral cortex of rats exposed to air-driven blast [24, 96] and
vasospasm has been found in pigs following exposure to chemical explosive-driven blasts [66].

Pathological characterization of blast injury also includes diffuse axonal injury, cerebral contusion, reactive astrocytosis [94, 96, 102, 103], and microgliosis [24, 96, 104, 105]. Brains of rats subjected to whole-body blast injury from a compressed air-driven shock tube have been shown to exhibit neuronal, glial, and myelin abnormalities similar to those observed with diffuse axonal injury [78], which is induced when rapid mechanical loading of the brain results in a viscoelastic response; tearing axons or damaging the axonal cytoskeleton [18]. Diffuse axonal injury independent of additional detectable clinical features is sufficient to induce coma [106] and loss of consciousness following exposure to blast without persistent overt clinical signs has been reported in troops diagnosed with bTBI [107]. Detectable injury due to blast has also been reported at the cellular level through ultrastructural changes in rat brains following neuronal swelling with cytoplasmic vacuoles and myelin deformation present in rat hippocampus following blast injury [95].

Glial scar formation has been shown to be detrimental to rebuilding CNS axonal structure following traumatic injury [28-34]. Communication, pain, sensory integration, and movement disorders that develop weeks to months after injury have all been linked to blast exposure [95, 108-110]. Other laboratories have shown increased astrocyte GFAP expression and localization in the hippocampus [111, 112], the primary motor cortex [112], posteriomedial cortical amygdala [112], sub-cortical white matter [113], and cerebellum [97]. Temporal development of the astrocytic response in previous studies of
blast-induced brain injury peaked at around 24-48 hours post injury [97, 112, 114] and extended into 2 weeks following injury in at least one case [111].

There are extensive reports on the immune response as a pathological outcome in both diffuse and focal classic models of traumatic brain injury [36, 115-119]. Additionally, rod-shaped microglia, thought to be involved in stripping of synapses [120], have been observed in reports of animal models of traumatic CNS insults [121, 122]. Increased microglial immunoreactivity and morphological changes in white matter [96], hippocampus [24, 96], cerebellum [96], brain stem [24, 96] consistent with brain injury have been reported following blast exposure as has widespread microgliosis and macrophage infiltration [104]. The temporal expression of microglial activity in other models of blast-induced brain injury was long-term in nature with the beginnings of detectable microglial morphology changes at approximately 1 day post injury [105] and extending through 1 month post injury [104]. Serum biomarkers associated with blast-induced secondary injury characteristics have been reported following blast injury and include GFAP and TNF-α [103, 123].
1.3 Blast Injury Models

Blast injury models can be separated based on two categories: shockwave source and enclosure type. There are two shockwave sources in widespread use today. Large animal (i.e., porcine) models use chemical explosives as the source of the shockwave [111, 124], while small animal (i.e., rat, mouse) models predominantly use compressed gas-driven membrane rupture [24, 96, 125, 126]. Blast injury models can also make use of a shock tube [24, 96, 111, 124-126], injure animals in a free-field setup in which there are no enclosures around the explosive charge or animal [127], or injure animals within an enclosed structure [111, 124]. Shock tubes are utilized to produce homogenous wave fronts from relatively small amounts of explosive than would be needed for a free-field explosion to achieve the same peak pressure at a given standoff distance from the blast source [128, 129]. While free-field explosions produce shockwaves which most closely resemble an ideal explosion [130], shock tubes have been shown to produce shockwaves very similar to Friedlander waveform [70, 124]. Building or vehicle type enclosures, such as those used by Bauman et al. create complex waveforms meant to simulate blast exposure while in a building or vehicle [124].

Many blast injury models exist and prior to the studies outlined in this dissertation, researchers had to choose the particular model which featured shockwave parameters they wanted to test; however other parameters such as standoff distance and reflection surfaces may not have been consistent between each model of choice. These differences make it difficult to compare results between models. The MBD makes it
possible to compare the results of shockwaves with different pressure-time signatures produced from different sources within the same device.
1.4 Specific Aims and Hypotheses

Mechanisms resulting in blast-induced brain injury have not been elucidated. The studies outlined in this dissertation sought to detect and characterize the difference in shockwaves of various sources within the same device. Once characterized, we sought to determine the extent of IgG extravasation caused by the increased positive phase duration of compressed air-driven shockwaves, the amount of astrocytosis and microgliosis caused by the increased positive phase duration of compressed air-driven shockwaves. We also sought to characterize the head rotation angles and velocities of rats exposed to compressed air- and oxyhydrogen-driven shockwaves in order to determine the extent of head rotation’s involvement in the injury.

**Overall Hypothesis:** *Compressed air-driven shockwaves will produce fundamentally different pressure-time signatures that will produce more brain injury as measured by brain injury markers than those of chemical explosives.* Due to the time necessary to produce complete membrane failure and due to the molecular interactions of the diatomic molecules that comprise the bulk majority of atmosphere, we expected the compressed air would take a longer time to exit the driver chamber due to intermolecular adhesion than would helium, thus elongating the positive phase duration. Together, we expected helium- and compressed air-driven modes of the McMillan Blast Device to exhibit longer durations than their chemical explosive-driven counterparts due to the time necessary for complete membrane failure. We expected compressed air- and helium-driven modes to necessitate greater amounts of driver gas to achieve a certain peak magnitude than would
be necessary if membrane failure or molecular interactions were not a factor. For this reason, we expected compressed air- and helium-driven shockwaves to contain greater energy than their chemical explosive-driven counterparts (i.e., RDX, oxyhydrogen) and that this greater amount of energy would cause more brain injury in rats exposed to them. Though the total amount of kinetic energy would differ between compressed air- and oxyhydrogen-driven blasts, we did not expect the total head rotation angle or velocities to differ between rats subjected to each mode of injury due to the tight confinement of the head by the plastic netting.

**Specific Aim 1:** To test the hypothesis that compressed air-driven shockwaves will exhibit longer positive-phase durations, greater impulse, and greater impulse difference at similar peak overpressures than shockwaves produced by compressed helium-driven membrane rupture or chemical explosives (RDX, cyclotrimethylenetrinitramine; oxyhydrogen, a 2:1 mixture of hydrogen and oxygen gasses). Due to the time necessary to produce complete membrane failure and due to the molecular interactions of the diatomic molecules that comprise the bulk majority of atmosphere, we expected the compressed air would take a longer time to exit the driver chamber than helium, thus elongating the positive phase duration. Together, we expected helium- and compressed air-driven modes of the McMillan Blast Device to exhibit longer durations than their chemical explosive-driven counterparts due to the time necessary for complete membrane failure. We expected compressed air- and helium-driven modes to necessitate greater amounts of driver gas than would be necessary if membrane failure or molecular
interactions were not a factor to achieve the same peak magnitude but with a longer duration. For this reason, we expected compressed air- and helium-driven shockwaves to contain greater energy than their chemical explosive-driven counterparts.

**Specific Aim 2:** To test the hypothesis that compressed air-driven shockwaves will produce more extensive blood-brain barrier compromise as evidenced by greater IgG extravasation in the brains of rats compared to oxyhydrogen-driven shockwaves of greater peak pressure but lesser energy. Vascular changes have been associated with blast-induced brain injury in humans [68, 77] and animal models [24, 96] of blast injury. Additionally, enclosures and structures which serve to elongate the positive phase duration of shockwaves have been reported to produce more extensive injuries from blast [68, 77, 124]. Therefore, we expected to see increased cerebrovascular compromise in response to the elongated positive phase of compressed air-driven shockwaves compared to that produced in response to the relatively shorter positive phase of oxyhydrogen-driven shockwaves.

**Specific Aim 3:** To test the hypothesis that compressed air-driven shockwaves will produce more reactive astrocytosis and microgliosis as evidenced by greater numbers of GFAP-(astrocytes) and Iba1-(microglia) positive cells with an activated morphology present in the brains of rats exposed to compressed air-driven shockwaves than in the brains of rats exposed to oxyhydrogen-driven shockwaves of greater peak pressure but lesser energy. Models utilizing enclosures capable of reflecting blasts waves and
producing longer positive phase durations have also been shown to exacerbate blast injuries [124]. Astrocytosis and microgliosis have been documented in animal models of blast-induced brain injury [24, 96, 124, 126]. We expected to see exacerbated markers of the longer-term secondary injury cascade in response to the elongated positive phase duration of compressed air-driven shockwaves compared to oxyhydrogen-driven shockwaves.

**Specific Aim 4:** To test the hypothesis that head rotation angle or average velocity will not significantly differ between compressed air- and oxyhydrogen-driven shockwave-exposed animals. Due to the fact that rat heads were tightly secured in plastic netting for all blast procedures, we expected the physical differences in compressed air- and oxyhydrogen-driven shockwaves to have a negligible effect on the total amount or velocity of rat head movement.
2. CHAPTER 2

A multi-mode shock tube for investigation of blast-induced traumatic brain injury

(Published in J. Neurotrauma, 28(1): 95-104; republication permission in Appendix I)

2.1 Introduction

Blast-induced mild traumatic brain injury (bTBI) has become increasingly common in the current conflicts due to the increased use of improvised explosive devices (IEDs) as well as advances in body armor and medical care resulting in improved survivability of blast injuries [69, 78, 85, 131]. The mechanisms by which non-impact blast exposure results in bTBI is incompletely understood and is the subject of much current investigation using both large and small animal models. While the majority of large animal models of blast injury utilize chemical explosives as the source of the blast wave [66, 132, 133], most small animal studies utilize compressed air-driven shock tubes [78, 95, 134].

Blast waves are created by the very rapid conversion of a solid or liquid into a gas [64]. The rapid expansion of the gas compresses the surrounding air to create a blast overpressure wave, which then decays exponentially and is followed by a relative vacuum, the underpressure wave. The pressure-time trace of an ideal blast wave is described by a Friedlander waveform (Fig. 1.1) [65].

Currently, there is uncertainty regarding the contribution of various components of a blast wave to bTBI. Most small animal blast injury studies examine the role of blast overpressure on test subjects by using peak overpressure as their measure of blast wave
intensity [74, 95, 126, 134, 135]. However, the peak overpressures used to induce bTBI have varied widely—ranging from 20 kPa to 340 kPa for whole body exposure in rats [90, 95], 150kPa for head-only exposure [93], and up to 10,000 kPa for direct brain exposure [91]. In addition to maximal overpressure, the damaging effects of a blast wave may also depend on the duration of the positive pressure wave, as well as a contribution from the negative pressure wave [136-138]. These characteristics are determined not only by the blast source itself, but also by distance from the blast and effects of reflections from walls or in a confined area such as a vehicle.

To examine the contribution of various components of the blast wave to bTBI, we designed and constructed the McMillan blast device (MBD, Fig. 2.1). This is a shock tube similar in design to the shock tube at the Walter Reed Army Institute of Research and Naval Medical Research Center in Silver Spring, MD [74, 126, 139]. The MBD is capable of utilizing four different modes to produce a shock wave: compressed air, compressed helium, oxyhydrogen and RDX (cyclotrimethylenetrinitramine, the main explosive component of C-4 plastic explosives). Each blast mode results in a distinct pressure-time trace. The principal goal of this study was to characterize the various blast modes produced by the MBD by evaluating six parameters of the resultant blast wave and pressure-time trace: shock wave velocity, positive phase magnitude, positive phase duration, positive phase impulse, negative phase magnitude, and the impulse difference between reflected and free-field pressures. Another goal of this study was to begin to examine the extent of brain injury produced by each of the blast modes at similar peak overpressures. This will allow evaluation of the potential contribution of different components of the blast wave to bTBI in small animal models.
Fig. 2.1. The McMillan Blast Device (MBD). (A) Photo of the entire device. The flanges at the adjacent edges between the compression and expansion chambers are lined with silicone gaskets to seal around a Mylar membrane. In compressed air- or compressed helium-driven mode, the Mylar membrane is naturally ruptured or is ruptured by a 4-point blade affixed to a pneumatic cylinder that is designed to induce complete membrane failure at a pre-determined pressure in the compression chamber. (B) The Mylar sheet is inserted between the compression and expansion chambers of the MBD. (C) For the explosive-driven modes (oxyhydrogen or RDX) a blast plate is inserted between the compression and expansion chambers. The plate contains a manifold for the hydrogen and oxygen, which flow into a flange facing the expansion chamber. The polyethylene bag is inserted over the flange and filled with the hydrogen and oxygen. (D) The reflected (face-on) pressure sensor (PCB model #113A24) embedded in the dorsal surface of the polyurethane rat model. (E) The free field (side-on) sensor (PCB model #137A22). (F) The anesthetized rat is fitted with a Kevlar vest (not shown) and inserted into a mesh netting support. This is then loaded into a shock tube insert. The netting is tightened and securely fastened to prevent rotational movement of the head during blast exposure. The insert is placed into a cutout in the MBD such that the rat is positioned laterally within the expansion chamber of the shock tube approximately 1 foot
from the open end, with the left side of the rat facing the blast source. Following blast exposure, the insert is rapidly removed and the rat is removed from the netting (RDX, cyclotrimethylenetrinitramine).
2.2 Materials and Methods

2.2.1 McMillan Blast Device

The MBD (Fig. 2.1) consists of a cylindrical steel tube, 12-inch internal diameter, separated into a 19 ft. expansion chamber and a 2.5 ft compression chamber. When in compressed air- or compressed helium-driven modes, a 10 mil (0.254mm) thick biaxially-oriented polyethylene terephthalate (Mylar®) membrane separates the two chambers (Fig. 2.1B) (Mylar A, Tekra Corp., New Berlin, WI, USA). In compressed gas-driven modes the compression chamber is filled with either compressed air obtained from an on-site air compressor or industrial grade compressed helium (Scott-Gross Company, Inc., Lexington, KY, USA) until the Mylar membrane spontaneously ruptures (compressed air-driven mode), or is manually ruptured at the desired load pressure (compressed helium-driven mode) by a 4-point blade affixed to a pneumatic cylinder.

In the oxyhydrogen-driven mode, a steel manifold was placed between the expansion and compression chambers (Fig. 2.1C). The manifold contained tubing for oxygen and hydrogen leading to a central flange on the side facing the expansion chamber. A thin polyethylene bag was attached to the flange and filled with a 2:1 mixture of gaseous hydrogen and oxygen. The oxyhydrogen mixture was ignited by a small cordite charge (Winchester Ammunition #209 ShotShell Primer, Olin Corporation, Clayton, MO, USA).

1 The McMillan Blast Device was named in honor of United States Army Cpl. William L. McMillan, III of Lexington, KY, USA who gave his life in service to his country on July 08, 2008 after his patrol was struck by an improvised explosive device.
In RDX-driven mode (not shown), an electric detonator (RockStar Electric Detonator, Austin Powder Co., Cleveland, OH, USA) was embedded in 1.4g of RDX (desensitized, Accurate Energetic Systems, LLC, McEwen, TN, USA). The two were then wrapped in a thin layer of latex and secured with electrical tape to create a single explosive unit, which was positioned in the center of the vertical axis of the expansion chamber six inches from the steel manifold. The explosive unit was detonated using an electronic blasting machine (Scorpion HB-SBS, E.I.T. Corp, Sunbury, PA, USA).

For each mode, the shock wave was recorded by face-on, reflected pressure (PCB Model #113A24, Fig. 2.1D) and free field, averaging (PCB Piezotronics, Inc. Model #137A22, Fig. 2.1E) sensors. The free field sensor was positioned inside the shock tube with the sensing element located 10 inches from the open end and the point facing towards the blast source. The reflected pressure sensor was installed in the dorsal surface of a solid polyurethane rat model (Fig. 2.1D). The model was then installed with the dorsal surface of the rat positioned such that the sensor faced toward the blast source. This sensor was also located inside the blast tube, 10 inches from the open end of the expansion chamber. During shockwave velocity recordings, two free field sensors were positioned inside the tube, separated by a distance of 12 inches. Data from each sensor was routed through a line signal conditioner (PCB Model #482A21) before being captured by a digital storage oscilloscope (MicroTrap VOD/Data Recorder, MREL Group of Companies, Ltd., Kingston, Ontario, Canada). Data were analyzed using MicroTrap 7.2 (MREL), DPlot v2.2.5.7, and Microsoft Excel 2007. Data were graphed using DPlot and PRISM v4.0.
2.2.2 Blast Wave Parameters and Data Analysis

The amount of compressed air, compressed helium, oxyhydrogen or RDX was adjusted so that the peak overpressure in each blast was approximately 120 kPa. Due to the oscillating nature of the pressure-time trace, a curve fit was utilized to determine the time at which the blast trace crossed the ambient pressure line (0 kPa). An 8th order least squares polynomial was found to be a superior fit and was fitted to the data using DPlot beginning at the peak pressure reported by the sensor (Fig. 2.2). Shockwave velocity was calculated based on the time required for the shockwave to travel between the two free-field sensors spaced 12 inches apart. Negative phase magnitude was the minimum value of the 8th order curve fit for a given pressure-time trace. Positive phase duration was calculated as the amount of time between the first substantial rise in pressure recorded for a given blast wave trace \(T_0\) and the time at which the 8th order curve fit crossed back over the ambient pressure line (0 kPa). Positive impulse is the integral of the pressure-time trace and is related to the linear kinetic energy contained in the blast wave. The impulse difference between reflected and free-field pressures (\(\Delta\) Impulse), also a measure of kinetic energy, was calculated by subtracting the positive impulse of the free-field sensor from that of the reflected pressure sensor [140].
Figure 2.2. Representative pressure-time traces are shown for each of the blast modes, with the free-field (side-on) sensor recordings shown in A, and reflected (face-on) sensor recordings shown in B. Eighth-order curve fits, indicated by a red line, were generated to smooth out the oscillations in the pressure-time traces to enable more accurate estimate of the time points at which the pressure traces crossed below the ambient pressure level (RDX, cyclotrimethylenetrinitramine; Comp. He., compressed helium; Comp. Air, compressed air).
2.2.3 Gas Content Following Blast

Using a gas other than air to fill the compression chamber of the MBD, or the combustion of explosives, may create a hypoxic environment that could compound injury data. Oxygen and carbon monoxide content were measured using a hand-held gas detector (iTX, Industrial Scientific, Oakdale, PA, USA). Immediately following detonation, the gas detector was placed within the MBD so that its sensing element was located where the test animal’s head would be. Oxygen and carbon monoxide levels were measured for 30 seconds after the detonation at which point the detector was removed and peak values were recorded.

2.2.4 Animal Blast Exposure

Male Sprague-Dawley rats were sedated (diazepam, 10mg/kg, i.p.) for transport to the blasting site. Rats were transported in individual cages and had access to food and water ad libitum throughout the course of the transport. Each cage was maintained in a climate-controlled vehicle until the rat was subjected to blast. Immediately prior to injury, rats were anesthetized (sodium pentobarbital, 50mg/kg, i.p.), fitted with a Kevlar vest designed to protect the thoracic organs [126], and placed into a mesh netting (Industrial Netting, Mineappolis, MN, USA) support and loaded into the MBD (Fig. 2.1F) laterally with their left side facing into the blast. Once loaded into the MBD, rat bodies were protected by a steel tube that surrounded their bodies but left their heads exposed to the blast (Fig 2.1F). Rats were subjected to compressed air- or oxyhydrogen-driven blasts of 100, 150 or 200kPa peak overpressure. Two rats were subjected to each
condition. Rats were euthanized with an overdose of sodium pentobarbital (150mg/kg, i.p.) three minutes following blast. Necropsy was performed on each rat. Thoracic and abdominal organs, including heart, lungs (including trachea), liver, spleen, kidneys, bladder, and gastrointestinal tract (esophagus at level of aortic arch through rectum), were removed, fixed in 10% buffered formalin, and evaluated by a veterinary pathologist. Rat brains were also removed and photographed (Fig. 2.8) during necropsy. Control rats were euthanized with an overdose of sodium pentobarbital and had their brains removed in the same way as injured rats. These studies were performed in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC). This was a preliminary study to determine the blast conditions associated with bTBI as well as possible damage to other internal organs.

2.2.5 Statistics

A one-way ANOVA followed a Student Newman-Keuls post-hoc analysis was used to compare the values for each parameter among blast modes. A one-way ANOVA followed by Dunnet’s post-hoc analysis was used to compare oxygen and carbon monoxide levels to control air. Statistics were performed using the PRISM v4.0 software package.
2.3 Results

2.3.1 Shockwave Velocity

The velocity of the shock wave was measured using two sensors, positioned 17 or 18 feet, respectively, from the diaphragm or detonation source. The velocity measured at this distance was approximately 470 m/sec for the RDX, compressed air, and compressed-helium modes, as compared to 520 m/sec for oxyhydrogen (Fig. 2.3).
Shockwave Velocity

Velocity (m/sec)

RDX  Air  Helium  H₂-O₂

*  **  **

Fig. 2.3. Shockwave velocity. Shockwave velocity was determined for each mode of the McMillan blast device as described in the methods section. This velocity was slightly greater in oxyhydrogen-driven mode than in the other three modes tested. Error bars represent standard deviation from the mean (*p<0.05, **p<0.01; n=3; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
2.3.2 Positive Phase

The blast conditions were adjusted such that the peak positive phase (overpressure) magnitudes, detected by the free field sensor, were approximately 120 kPa. These peak overpressures did not differ significantly for each of the four modes examined (Fig. 2.4). For the reflected pressure, the maximal overpressure for the RDX-, compressed helium- and oxyhydrogen-driven modes was similar, and slightly lower for the compressed air-driven mode (Fig. 2.4).
Fig. 2.4. Mean positive magnitude for both sensors in each blast mode. Error bars represent standard deviation from the mean (*p<0.05, **p<0.01, n=6; RDX, cyclotrimethylenetrinitramine; H₂O₂, oxyhydrogen).
Positive phase duration reported by the free field sensor was significantly shorter with RDX as compared to compressed air-, compressed helium- and oxyhydrogen-driven blasts (Fig. 2.5). Both compressed helium- and oxyhydrogen-driven blasts had shorter positive durations than compressed air (Fig. 2.5). Positive phase durations reported by the reflected pressure sensor were longer than those observed with the free field sensor, and were significantly greater in duration in both compressed air- and oxyhydrogen-driven blasts than in RDX- and compressed helium-driven blasts (Fig. 2.5).
Fig. 2.5. Mean positive phase duration for both sensors in each blast mode. Error bars represent standard deviation from the mean (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=6; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
Positive impulses, calculated from free field sensor data were lowest for RDX, slightly greater for compressed helium and oxyhydrogen, and greatest for the compressed air driven blasts (Fig. 2.6). The positive impulses calculated from reflected pressure sensor data taken during compressed air-, compressed helium- and H$_2$-O$_2$-driven blasts were all higher than those of RDX-driven blasts (Fig. 2.6). The overall pattern was largely similar to that observed with the free field sensor, with the impulse for compressed air being much greater than that observed with the other blast modes where compressed helium was slightly greater than oxyhydrogen and RDX-driven blast waves (Fig. 2.6).
Fig. 2.6. Mean positive impulse for both sensors in each blast mode. Error bars represent standard deviation from the mean (***p<0.001, n=6; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
2.3.3 Negative Phase

The peak negative phase magnitude, detected by the free field sensor, was similar for the RDX, compressed air, and compressed helium modes but substantially less for oxyhydrogen (Fig. 2.7). For the reflected pressure sensor, similar peak underpressures were observed for RDX, compressed helium, and oxyhydrogen, while the magnitude of the peak negative pressure was much greater with compressed air (Fig. 2.7).
Fig. 2.7. Negative phase mean peak magnitude for the free-field (white bars) and reflected (gray bars) pressure sensors. Error bars represent standard deviation from the mean (**p<0.01, ***p<0.001; n=6; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
The duration of the negative phase was difficult to accurately estimate from the pressure-time histograms. The negative phase duration and impulse were therefore not calculated.

2.3.4 Impulse Difference

At similar peak overpressures, the difference between the impulse reported by the reflected and free-field sensors [140] was greatest for compressed air-driven shockwaves, followed by helium, and lowest for the oxyhydrogen- and RDX- driven blasts (Fig. 2.8).
Fig. 2.8. Impulse difference. The difference in impulse was calculated by subtracting the positive impulse obtained using the free-field sensor from that recorded with the reflected pressure sensor. Error bars represent standard deviation from the mean (***p<0.001, n=6; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
2.3.5 Gas Content Following Blast

Oxygen content was reduced by approximately 75% following helium-driven blasts and moderately reduced following oxyhydrogen-driven blasts (Fig. 2.9). Carbon monoxide content was very high following oxyhydrogen-driven blasts and above acceptable levels following RDX-driven blasts, although the increase in carbon monoxide levels following RDX-driven blasts was not statistically significant (Fig. 2.9). The elevated carbon monoxide levels observed following oxyhydrogen-driven blasts are the result of combustion of the polyethylene bag used to contain the oxyhydrogen gas prior to detonation, as there is no source for carbon in a hydrogen-oxygen condensation reaction. Combustion of polyethylene results in high levels of carbon monoxide [141]. Similarly, the slight increase observed following RDX is thought to result from combustion of the latex and electrical tape used to secure the RDX prior to detonation.
Fig. 2.9. Gas content following blasts. Oxygen (A) and carbon monoxide (B) levels within the McMillan blast device were measured following blasts of approximately 120kPa. There was a substantial decrease in oxygen levels following helium-driven blasts, and a more modest decrease following oxyhydrogen-driven blasts. Carbon monoxide levels were greatly elevated following oxyhydrogen-driven blasts, as a result of combustion of the polyethylene bag used to contain the gases prior to detonation. The slight increase in carbon monoxide seen following RDX-driven blasts was not statistically significant, but is also thought to result from combustion of the latex and electrical tape used to secure the RDX prior to detonation. Error bars represent standard deviation from the mean (**p<0.01, n=3; RDX, cyclotrimethylenetrinitramine; H₂-O₂, oxyhydrogen).
2.3.6 Gross Brain Pathology

The brain surface vessels of rats exposed to 100 kPa peak overpressure shockwaves appear enlarged and more prominent as compared to those observed in a control (non-blast exposed) rat brain (Fig. 2.10). At 150 kPa blast overpressure the blood vessels appear larger and hematomas are evident. This is even more prominent at 200 kPa. At each blast overpressure, the external gross brain pathology is more pronounced with compressed air as compared to oxyhydrogen driven blasts (Fig. 2.10). Necropsy did not reveal blast-related pathology to thoracic and abdominal organs in any of the treatment groups.
Fig. 2.10. Gross brain pathology. Representative photographs of the rat brains following exposure of the rats to compressed air-driven and oxyhydrogen-driven blasts of various peak overpressures. Blast exposure resulted in larger blood vessel diameters and hematomas (white arrows), which were more prominent with increasing peak overpressures. The vascular damage was also more prominent with compressed air-compared to oxyhydrogen-driven blasts of similar peak overpressures.
2.4 Discussion

Blast-induced TBI in military and civilian populations results from shock waves produced by high-energy chemical explosives [78, 85]. This can be modeled using chemical explosives in the open field, often with large animals as subjects [132, 142, 143]. Alternatively, shock tubes offer several advantages for investigations of the physiological effects of blasts, particularly with small animals [67, 72]. Shock tubes are designed to focus the energy from the blast wave source in a linear direction thus maximizing the amount of blast energy that impacts the test subject and decreasing the variability in the blast wave itself. In contrast to free-field explosions, the velocity and pressure of the shock wave does not decay exponentially along the distance of the shock tube [72]. Also, smaller quantities of explosives are required to produce target peak overpressures [66].

Shock tubes typically consist of a tube in which a high-pressure gas, the driver gas, is separated from a low-pressure gas, the driven gas, by a diaphragm. The diaphragm can be ruptured by the pressure of the driving gas, mechanically, or by an explosive charge using a combustible mixture of gases. Following the rupture of the diaphragm, the resultant pressure waves compress into a shock wave that travels through the driven gas at a supersonic velocity. In the present study the driver gas was compressed air, compressed helium, a mixture of oxygen and hydrogen, or the chemical explosive RDX. The driven gas was atmospheric air.

Compressed air-driven shock tubes have been used to examine the effects of blast waves on small animals since approximately 1949 [73, 144]. By varying the size of the
high-pressure chamber, the positive phase duration can be altered for a given maximal overpressure [72, 145]. Air-driven shock tubes are the model most widely used for experimental bTBI studies [72, 95, 126, 133, 134, 139]. However, the pressure-time trace from air-driven shock tubes is ‘flatter’ than the peaked waves resulting from high explosives [145]. As a result of the flattening of the peak prior to decay of the shockwave, the duration of the overpressure wave was greater for compressed air as compared to other driving modes in the present study. Also, the peak overpressures attained with compressed air plateau with increasing pressure in the compression chamber [72], making it difficult to achieve a wide range of peak overpressures. In addition, air-driven shock tubes do not model other components of a chemical blast including acoustic, thermal, optical, and electromagnetic components [77]. Thus, while compressed air driven shock tubes are convenient and certain properties of the resultant shockwave are modifiable, it is also important to recognize the differences in the shockwave resulting from compressed air as compared to that produced by a chemical explosion.

Compressed atmospheric air is a mixture of mostly diatomic nitrogen, followed by diatomic oxygen and, to a much lesser extent, water vapor and other trace gasses. Due, in part, to intermolecular forces strengthened during compression, compressed air fails to expand as quickly as would an ideal gas when the membrane is ruptured. Use of a light gas, such as helium, improves the performance of shock tubes due to the increased speed of sound in helium as compared to air, resulting in a lower driver-to-driven tube ratio [146, 147]. This is consistent with results obtained in the present study, where helium produced a sharper overpressure peak and shorter overpressure duration as
compared to compressed air. However, a disadvantage is the expense of helium and the large amounts required to pressurize the driver tube [146]. Additionally, following shock tube blasts, the driver gas replaces all or part of the driven gas. Oxygen monitoring experiments revealed a 75% reduction in oxygen content within the shock tube following helium-driven blasts.

The variability (standard deviation) of the peak overpressures obtained with helium was greater than that observed for other blast modes. For compressed-air driven blasts, natural rupture of the Mylar membrane produces a shockwave with a peak overpressure of approximately 120 kPa. To produce a helium-driven shock wave of equal peak overpressure, the Mylar membrane had to be mechanically ruptured at a lower compression chamber pressure. This is because helium, being a lighter gas, expands more rapidly than air following membrane rupture. The greater variability observed in recordings from helium-driven blasts may be due to variations associated with the mechanical rupture of the Mylar membrane at the desired compression chamber pressure.

RDX (cyclotrimethylene trinitramine) is the major component of plastic explosives widely used by the military and in improvised explosive devices [148, 149]. Although use of RDX provides an accurate representation of the chemical explosives encountered in warfare, experimentation with explosive nitroamines including RDX is not without significant drawbacks. In addition to the cost of the explosive and detonators consumed in each blast, explosive nitroamines require specialized equipment for their proper transport and use including explosive storage containers, dynamos or “shot boxes” for detonator ignition and often a separate transport vehicle. Using explosive nitroamines
in the United States requires federal licensure that can be costly to obtain and requires at least two years working experience with explosives to obtain, in addition to individual state requirements. In addition, RDX in its undetonated form is a suspected carcinogen [150] and the byproducts of RDX detonation contain potent vasodilators similar to glycercyl trinitrate tablets, thus presenting a risk of developing a nitric oxide tolerance to handling personnel. These disadvantages make RDX unsuitable as a driving source for investigations involving small animals.

When detonated, oxyhydrogen undergoes a chemical reaction that yields energy much like a RDX detonation, but the chemical byproduct is water vapor. Additionally, compressed hydrogen and oxygen can be purchased without a license, stored separately and only combined when inside the MBD. The oxyhydrogen-driven blasts are also relatively inexpensive, as small amounts of oxygen and hydrogen are utilized and other expenses include the chordite charge (shotgun primer) and a polyethylene bag to contain the gasses within the device until detonation. High carbon monoxide levels following oxyhydrogen-driven blasts are likely due to burning of the polyethylene bag. With both the helium and oxyhydrogen-driven blasts, it is important to remove the experimental animal from the shock tube within a few seconds following blast exposure to prevent consequences of prolonged exposure to helium or carbon monoxide.

The pressure-time traces produced by oxyhydrogen were similar to RDX in many respects. However, oxyhydrogen had a faster average shockwave velocity as compared to RDX and compressed air and helium. This may reflect the heat generated during the oxyhydrogen explosion, which results in a greater expansion of the gas and allows the
shockwave to travel faster. Heating of the driving gas is one method used to improve shock tube performance [146]. Overall, the results demonstrate that the pressure-time traces produced by oxyhydrogen most closely resemble those generated by RDX as compared to compressed air and helium.

Blast components that contribute to bTBI are largely unknown. Previous studies have largely focused on the maximal overpressure as the critical factor [95, 126, 134, 135]. However, the peak overpressures used to induce bTBI have varied widely—ranging from 20 kPa to 340 kPa for whole body exposure in rats [90, 95], 150kPa for head-only exposure [127], and up to 10,000 kPa for direct brain exposure [91]. As noted by Ling [77], the assumption that bTBI is dependent only upon the peak overpressure may not be valid.

Although there is a relationship between peak pressure and lethality in sheep [151], duration of the overpressure phase and the positive impulse (integral of overpressure x duration) are also known to influence the extent of lung injury [67]. A model of the underpressure component of the blast wave can result in similar lung damage to that produced by overpressure [152]. Of particular relevance in the present study is that compressed air differed substantially from the other blast modes in having a greater positive impulse and Δ impulse at similar maximal overpressures. This suggests the possibility that a compressed air-driven shockwave may be more damaging than a chemical explosive-driven shockwave of similar peak overpressure. In the present study, the vascular damage on the brain surface was more pronounced following compressed air as compared to oxyhydrogen-driven blasts of similar peak overpressures. The
hematomas observed in the present study are consistent with those observed in both military personnel and civilians following blast exposure and in previous studies utilizing animal models of bTBI [98, 101, 153, 154]. A more thorough quantitative evaluation of gross and microscopic brain pathology following exposure of rats to the various blast sources is ongoing.

The blast wave components contributing to bTBI are not well understood, but may be quite different from those causing damage to air-filled organs such as lung [77]. Using a single blast source, it is difficult to determine the blast wave characteristics that contribute to injury. The MBD can utilize a variety of driving sources (compressed air, compressed helium, oxyhydrogen, RDX) to produce shock waves of similar maximal overpressure. The results demonstrate that these different blast modes differ substantially in their pressure-time traces at similar peak overpressures and that these differences may influence the extent of brain injury produced by each blast mode.

Compressed air-driven blasts had a much longer positive duration, impulse, and dynamic energy as compared to the other blast modes, and also resulted in greater vascular damage as compared to oxyhydrogen. Helium-driven shockwaves more closely resembled those produced by RDX, but by replacing air within the expansion chamber of the shocktube created a hypoxic environment. Oxyhydrogen-driven shockwaves closely resembled those resulting from RDX, but produced high levels of carbon monoxide resulting from combustion of the polyethylene bag. For both helium- and oxyhydrogen-driven blasts, rapid removal of animals following blast exposure is necessary to prevent damage resulting from the hypoxic environment within the shock tube. This multi-mode
shock tube will enable comparison of the pressure-time signature produced using each blast mode with the extent of brain injury in small animal models, facilitating evaluation of blast wave components contributing to bTBI.
3. CHAPTER 3

Vascular damage in response to different pressure-time signatures

3.1 Introduction

Secondary injury mechanisms associated with TBI pathology are often a significant source of damage and dysfunction in addition to primary mechanical trauma. One significant consequence of sustaining a TBI is vascular damage. Physical disruption of brain vasculature can lead to hematoma and hemorrhage within the cranial vault.

Vasogenic edema caused by compromise to the blood-brain barrier (BBB) giving rise to leaky blood vessels following TBI can occur alone and often leads to significant accumulation of extravasated fluid within the brain parenchyma [155]. Further edema through a vasogenic response to cytotoxic mechanisms leads to a cyclical downward progression of the injured brain’s ability to recover [23]. Regional changes in blood flow and changes in blood-brain barrier permeability have been reported in rodent models of diffuse [51, 156, 157] and focal [45] models of traumatic brain injury. Current treatments are aimed at reducing intracranial pressure and, thus, increasing cerebral perfusion pressure and breaking the cycle of edema, however these treatments work through reducing osmotic pressure and do not treat the source of the BBB problem. Nevertheless, it is important to determine the contribution of BBB compromise in models of traumatic brain injury.

Reported peak pressures necessary to produce blast injury have varied greatly from 20kPa [90] for whole-body exposure to 20,000kPa for direct brain exposure to
shockwaves [91]. These reports suggest there are additional components of the shockwave that can contribute to the brain injury from blast. We developed and characterized a rat model of blast injury that utilizes a multi-mode shock tube (McMillan Blast Device; MBD) to produce shockwaves with different unique pressure-time signatures [70]. We examined the brains of rats exposed to compressed air-driven shockwaves of 175kPa peak target overpressure and oxyhydrogen-driven shockwaves of 250kPa peak target overpressure for evidence of blood brain-barrier compromise at 3 hours post blast exposure. This time point was chosen to coincide with the time at which maximal IgG extravasation has been reported to occur in other models of blast-induced traumatic brain injury [24, 96]. Previous studies of blast-induced brain injury have reported the most substantial IgG extravasation at 3 hours post injury. Though oxyhydrogen-driven shockwaves exhibited a higher peak overpressure than the compressed air-driven shockwaves, the impulse (and, thus, energy) delivered by a compressed air-driven shockwave was greater than that of an oxyhydrogen-driven shockwave. We also compared extravasated IgG levels to the actual pressure and impulse to which the rats were exposed by correlation analysis in an effort to determine the contribution of positive phase duration on blood-brain barrier compromise. The results of this study provide support for the importance of the positive phase duration on blast-induced brain injury.
3.2 Materials and Methods

3.2.1 Animal Use

All animal use procedures utilized in this study were performed in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Animals had access to food and water ad libitum throughout the course of this study. Adult (8-week), male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN, USA) were separated into individual animal cages, sedated (diazepam, 4mg/kg, i.p.), and transported via climate controlled passenger van from the animal housing facility to the blast site. At the blast site, the animals were maintained in a climate-controlled room with adequate lighting for the duration of their time at the blast site. Immediately prior to injury, animals were deeply anesthetized (ketamine 60mg/kg, xylazine 7.5mg/kg, i.p.), fitted with a Kevlar vest, placed within polyethylene netting (Industrial Netting, Minneapolis, MN, USA), and loaded into the McMillan Blast Device (MBD) [70] in the prone position with their left side facing the shockwave source. Animals were subjected to compressed air- or oxyhydrogen-driven blasts of 175kPa or 250kPa peak overpressure, respectively. Animals were removed immediately following blast in order to prevent confounding effects of hypoxia and acute carbon monoxide exposure [70]. Following blast injury, animals were returned to their individual cages with heat support (Deltaphase Isothermal Pads, Braintree Scientific, Braintree, MA, USA) to prevent anesthesia-induced hypothermia. Animals were maintained in dorsal recumbency for the post-blast anesthesia period and were periodically monitored for respiration. Cardiac activity was monitored via thoracic palpation. Due to the fact that
headache is known to occur in humans exposed to blast, rats were weighed and given carprofen (7mg/kg, i.p.) every 12h after injury until their scheduled euthanasia time. Once recovered from anesthesia, all animals were placed in clean individual cages and transported back to the University of Kentucky animal facility where they remained in their individual cages until euthanasia.

3.2.2 Euthansia and Tissue Collection

At 3h post injury animals were administered an overdose of sodium pentobarbital (150mg/kg, i.p.). This time point was chosen based on known peak BBB opening time following other modes of traumatic brain injury [24, 96, 126]. Once animals were deeply anesthetized, thoracotomy was performed and whole blood was collected via syringe from the inferior vena cava. Animals were then transcardially perfused with 200mL of phosphate-buffered saline (PBS), followed by 200mL of 4% paraformaldehyde in PBS. Animals used for Western blot analysis were perfused only with PBS. After perfusion, animal brains were removed and photographed for evidence of hematoma and petechial hemorrhage. Brains used for immunohistochemistry were placed in 4% paraformaldehyde in PBS overnight at 4°C, followed by three days in 30% sucrose in PBS at 4°C for cryoprotection, after which they were embedded in OCT compound and stored at -80°C until sectioning. Brains used for Western blot analysis were separated into cerebrum and brainstem/cerebellum sections by severing the cerebral peduncles and brainstem-diencephalon connections. The cerebrum was further subdivided by a midsagittal bisection creating left and right cerebrum samples. The left and right
cerebrum samples were further subdivided into dorsal and ventral sections by horizontal cuts just ventral to each hippocampus. These cuts resulted in four cerebrum samples for each animal: ipsilateral ventral cortex (IVX), ipsilateral dorsal cortex (IDX), contralateral ventral cortex (CVX), and contralateral dorsal cortex (CDX) (Fig.3.4B). Brainstem (BS) and cerebellum (CBM) were separated by severing the cerebellar peduncles. Each sample was wrapped in aluminum foil, flash frozen in powdered dry ice, and kept frozen at -80°C until processing for protein.

3.2.3 Immunohistochemistry

Brains were sectioned at 40µm thickness on a freezing stage sliding microtome (Microm HM 440 E, Microm, Walldorf, Germany). Sections were collected in Tris-buffered Saline (TBS). Sections representative of four different anterior-posterior levels were matched across animals and blocked for 1 hour in 1% horse serum in TBS and treated in primary antibody in 1% horse serum in TBS for 1 hour at room temperature. Antibody against immunoglobulin G (IgG, Goat ant-Rat IgG-Biotin, 1:5,000, Jackson Immunoresearch, West Grove, PA, USA) was diluted in TBS with 1% horse serum and applied to the sections overnight at room temperature. Biotinylated antibody treated slices were treated with streptavidin-conjugated horseradish peroxidase (1:5,000, Jackson Immunoresearch). Presence of antibody reactivity was detected via enzymatic oxidation of 3,3’-diaminobenzidine (DAB, Jackson Immunoresearch). Following staining, slides were mounted on to slides (Fisher SuperFrost Plus, Fisher Scientific, Waltham, MA, USA) and mounted with Permount (Fisher Scientific) and coverslips. Slides were
imaged on an Olympus AX 80 microscope. IgG images were analyzed using Image J software for the percentage area reactive to IgG. Of the four anterior-posterior levels matched between all animals, the level with the most consistent IgG staining between all animals in a given treatment group was chosen for the Image J analysis. Images presented in Fig. 3.2 were subjected to binary isodata-algorithm threshold analysis (Fig. 3.3A). Any pixel that crossed threshold was assigned the color black and any pixel that failed to cross threshold was assigned the color white. Percentage brain section area positive for black pixels was recorded and averaged. Areas counted as IgG positive and/or part of the section area but that were part of the ventricles were subtracted from the total.

3.2.4 Western Blotting

Frozen brain tissue was weighed, covered with 150µl RIPA Buffer (Radio-immunoprecipitation Assay Buffer) and allowed to thaw on ice. Thawed tissue was chopped, placed in a dounce homogenizer with cell lysis buffer (2ml per g of tissue), and homogenized by 20 passes in the homogenizer. Resulting lysates were purified by centrifugation (2X 15min at 13.2 rcf). Protein concentrations were quantified by bicinchoninic acid (BCA) Protein Assay (Thermo Scientific). Lysates were separated by SDS-PAGE (10 % Bis-Tris, Invitrogen) using MOPS Running Buffer (Invitrogen), transferred to nitrocellulose membranes via semi-dry protein transfer (Trans-Blot Turbo Transfer System, Bio-Rad, Carlsbad, CA, USA). Membranes were blocked in Tris-buffered Saline (TBS) containing 5% (w/v) non-fat powdered milk for one hour at room
temperature to prevent non-specific antibody-protein interaction. Once blocked, membranes were treated with TBS containing 0.05% Tween-20 (TTBS) and primary antibody (1:1,000, Sigma) overnight at 4°C. Membranes were washed 3 times for 5 minutes with TTBS, then treated with TTBS containing the appropriate secondary antibody conjugated to an infrared fluorophore (1:5,000, Rockland). Following secondary antibody treatments, membranes were washed 3 times for 5 minutes with TTBS, then imaged on an Odyssey Scanner (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed for relative band intensity on the Image Studio software (Li-Cor).

3.2.5 Statistical Analysis

Data presented in Figs. 3.3 and 3.4 was analyzed by One-Way ANOVA. The Student Newman-Keuls post hoc analysis was selected for comparisons between treatment groups due to its ability to protect against both Type I and Type II statistical error. A Two-Tailed Pearson correlation was used in combination with linear regression analysis to examine the extent of IgG extravasation due to pressure or impulse presented in Fig. 3.5.
3.3 Results

3.3.1 Surface Vessel Damage and Hematoma Following Blast Exposure

Gross examination of the brains of rats exposed to both oxyhydrogen- and compressed air-driven shockwaves revealed surface vessel damage (Fig. 3.1). The brains of rats exposed to compressed air- and oxyhydrogen-driven shockwaves exhibited large scale hematoma and petechial hemorrhage compared to those from sham-exposed animals (Fig. 3.1). The hematoma were centered on the ventral surface of the brain surrounding the median eminence (Fig. 3.1). Hematoma and petechial hemorrhage were evident on the ventral surface of the brains of 5 of 6 rats exposed to compressed air-driven shockwaves, and 3 of 6 rats exposed to oxyhydrogen-driven shockwaves (Fig. 3.1). While hematoma were evident on the ventral surface of the brains of rats exposed to both modes of the MBD, these hematoma were less extensive and the petechial hemorrhage was less evident in the brains of oxyhydrogen-injured rats (Fig. 3.1).
Fig. 3.1. Qualitative examination of vascular damage can be observed in the images above depicting hematoma and petechial hemorrhage on the ventral surface of brains taken at 3 hours post-injury from rats exposed to oxyhydrogen- or compressed air-driven shockwaves. Note larger hematoma and more extensive petechial hemorrhage (white arrows) in the brain from a compressed air-driven shockwave-exposed rat. Images are representative of six rats per treatment group.
3.3.2 Histological Evidence of Blood-Brain Barrier Damage Following Blast Exposure

In addition to gross pathological evidence of vascular compromise due to blast, brains of rats subjected to oxyhydrogen- and compressed air-driven shockwaves were examined for histological evidence of blood-brain barrier compromise. Brains of rats subjected to compressed air-driven shockwaves showed IgG staining that was specific to the inferior cerebrum on the left side; the side first impacted by the shockwave (Fig. 3.2). Representative slices taken from rat brains subjected to sham injury or to oxyhydrogen-driven shockwaves exhibited no bilaterally asymmetric IgG staining (Fig. 3.2). Periventricular and median eminence IgG staining is normal in uninjured animals as the blood-brain barrier is incomplete in these areas.

Images presented in Fig. 3.2 were subjected to binary isodata-algorithm threshold analysis (Fig. 3.3A) to determine which areas of the images crossed threshold. Areas determined to have crossed threshold were considered IgG positive. Quantification of the percentage area staining positive for IgG extravasation revealed a significant increase in IgG staining in brain sections from animals subjected to compressed air-driven shockwaves, but not sham- or oxyhydrogen-exposed animals (Fig. 3.3B). Combined with hematoma and petechial hemorrhage data presented in Figs. 3.1 and 3.2 we can deduce that there is significant blood-brain barrier compromise produced by compressed air-driven shockwaves that is not present in animals exposed to sham- or oxyhydrogen-driven shockwaves. Differences in IgG extravasation between shockwave sources were not detectable by Western blot (Appendix B: Western blot analysis of blood-brain barrier compromise).
Figure 3.2. Histological evidence of blood-brain barrier compromise following compressed air-driven shockwave exposure. IgG extravasation can be visualized in the inferior left (side of the shockwave source) side of the brains in 5 of 6 rats exposed to compressed air-driven shockwaves. This region specific staining is not evident in the brains of rats exposed to sham injury or oxyhydrogen-driven shockwaves. Brain sections are each from a different animal euthanized at 3 hours post-injury and are presented in order from least severe to most severe IgG staining. n=6 per treatment group.
Fig. 3.3. Quantification of IgG Immunoreactivity. Sections stained with anti-IgG antibody were analyzed for percent area exhibiting IgG immunoreactivity (A). The percentage area of the slice that was positive for IgG immunoreactivity was averaged. Brains from rats exposed to compressed air-driven shockwaves exhibited more area immunoreactive to IgG than sham- or oxyhydrogen-driven blast-exposed rats at 3h post-injury (B). One-way ANOVA with Student Newman-Keuls post-hoc analysis, *p<0.05, n=6 per treatment group.
3.3.3 Shockwave Energy Dose Response

Rats were exposed to compressed air-driven shockwaves of 125kPa, 150kPa, or 175kPa peak target pressure. Western blot analysis showed a positive trend of increasing IgG levels present in the IVX region with increasing compressed air-driven peak pressure (Fig. 3.4 C). There was no positive trend IgG levels with increasing pressure in either the CBM or BS regions (Fig. 3.4 D & E). While sham IgG levels were relatively constant, there was substantial variability in the IgG levels throughout all regions sampled at all pressures (Fig. 3.4 C-E).
Fig. 3.4. Blast overpressure dose response. A. Representative bands from Western blots used to quantify IgG levels in the ipsilateral ventral cortex (IVX), cerebellum (CBM), and brain stem (BS). Samples were separated by electrophoresis in an order alternative to the one presented here to decrease inter-lane variability, however all blots shown here were imaged concurrently, and cropped to be repositioned as shown here. No other image modifications were made. B. Diagram showing location of cuts made to separate cerebrum into ipsilateral ventral cortex (IVX), contralateral ventral cortex (CVX), ipsilateral dorsal cortex (IDX), and contralateral dorsal cortex (CDX). C. Average IgG levels compared to actin levels in the IVX of animals exposed to increasing blast overpressure. D. Average IgG levels compared to actin levels in the CBM of animals exposed to increasing blast overpressure. E. Average IgG levels compared to actin levels in the BS of animals exposed to increasing blast overpressure. One-way ANOVA, n=4 per treatment group.
3.3.4 IgG Extravasation as a Function of Pressure or Impulse

Comparison of IgG:Actin ratios as measured by Western blot analysis revealed a positive correlation between the total amount of IgG present in the IVX region and the amount of peak overpressure to which individual rats were exposed (Fig. 3.5 C). Additionally, there appears to be a threshold effect at approximately 150kPa peak pressure. Animals exhibiting IgG:Actin ratios above 0.1 were mostly exposed to a peak overpressure of 150kPa or greater (Fig. 3.5 A). There was no positive correlation between the impulse to which the animals were exposed and the IgG:Actin ratios these animals exhibited either when considered within an individual shockwave source (Fig. 3.5 B) or regardless of source (Fig. 3.5 D). No statistically significant relationship between peak overpressure or impulse exposure and IgG extravasation was found in the cerebellum (Appendix C) or the brain stem (Appendix D).
Fig. 3.5. IVX IgG:Actin ratios as a function of peak overpressure and impulse exposure.  
A. Peak blast overpressure exposure vs. IgG:Actin ratio separated by shockwave source.  
There appears to be a threshold effect whereby animals exhibiting IgG:Actin ratios above 0.1 were also mostly exposed to peak overpressures of 150kPa or greater.  
B. Peak blast impulse exposure vs. IgG:Actin ratio separated by shockwave source.  
There was no positive effect of impulse on IgG extravasation for animals exposed to compressed air- or oxyhydrogen-driven shockwaves.  
C. Peak blast overpressure exposure vs. IgG:Actin ratio.  
A positive correlation between the amount of IgG as detected by Western blot analysis and the peak overpressure to which the animals were exposed was evident.  
D. Peak blast impulse exposure vs. IgG:Actin ratio.  
Though there was a general positive trend in the relationship between impulse and IgG extravasation, the relationship was not statistically significant.  
Pearson correlation, two-tailed.  
\( n = 6 \) or 19 (A & B) or \( n = 25 \) (C & D) per treatment group.
3.4 Discussion

Hematoma has been reported in both human case studies of blast injury [98-101] and animal models of bTBI [158]. Overt presence of hematoma precludes a diagnosis of mild TBI [159], however the cognitive sequelae following blast exposure of magnitudes utilized in this study are more similar to a mild injury [160, 161]. Mice exposed to blasts of similar peak overpressure exhibited mild deficits on spatial recognition tasks, Rotarod measurements of motor coordination [160]. Furthermore, food consumption and exercise performance studies have suggested mild behavioral changes associated with memory, motor, and motivational activities [161]. Regardless of behavioral findings of other researchers using blasts of similar overpressure to those utilized in this study, the size of hematoma present in compressed air-driven shockwave-exposed animals is substantial and, when accounting for size scale, would result in more significant behavioral deficits in humans with hematomas of similar size. Differences in shockwave physics between the sources used in this study could account for the differential hematoma profile. Given the dependency of the brain on cerebrovasculature, a disruption in the blood supply of the magnitude that would result from hematoma of this size would cause significant behavioral problems in individuals exposed to blast [125, 162, 163].

Blood-brain barrier disruption due to compressed air-driven shockwave exposure was centered around the inferior cortex on the side of the brain that was first impacted by blast. This is in contrast to what has been described in previous models of blast overpressure injury where IgG staining was detected in the contralateral cortex and cerebellum following blast injury [24, 164]. Interestingly, these other models of blast
injury utilized rigid animal holding mechanisms that could have contributed to the brain injury through tertiary means [24, 164]. Given the discrepancy between the IgG staining distribution in the present study and in previous studies [24, 164], this may represent a unique incidence of primary blast-induced blood-brain barrier compromise devoid of secondary or tertiary injury influences.

Though we were unable to generate a dose response effect in terms of significantly higher amounts of IgG extravasation with increasing blast overpressure detected by Western blot analysis, tissue IgG content in the IVX was found to positively correlate with the peak pressure to which the animals were exposed. IgG extravasation, hematoma and petechial hemorrhages were more exaggerated in animals exposed to compressed air-driven shockwaves than in animals exposed to oxyhydrogen-driven shockwaves, even though the average peak overpressure of compressed air-driven shockwaves was approximately 75kPa lower than that of oxyhydrogen-driven shockwaves. Taken together, these data suggest that both peak pressure and positive phase duration play a role in the cerebrovascular response to blast injury.

To the best of our knowledge, this study is the first example of shockwave source-specific injury characteristics to date. Previous studies in rats and larger animals have reported blood-brain barrier disruptions or vascular changes following blast overpressure [24, 164] or chemical explosive-induced brain injury [124, 127], however the cerebrovascular response to shockwaves from multiple sources with varying pressure-time signatures was not previously examined. Overpressure durations in previous bTBI studies vary from 0.3 ms to 52 ms [95, 165], while overpressure durations in the present
study varied from 3.2 ms to 5.3 ms. When compared to the vast overpressure durations reported in previous bTBI studies, our data suggests a small increase in positive phase duration can have a substantial impact on the cerebrovascular response.

Certain environments, such as building or vehicle enclosures, common in battlefield situations could give rise to increased positive phase duration exposure and could result in exacerbated injury characteristics from a blast that may not have caused such injury in an open environment. The findings of this study suggest that positive phase duration should be considered when evaluating the extent and type of brain injuries sustained due to blast. Additionally, given the results presented in this study, in addition to reducing the amount of blast pressure allowed through protective armor, engineers and designers of blast wave mitigation technologies may want to focus their efforts on shortening the positive phase duration of the shockwave as well.
4. CHAPTER 4

Evaluation of the differential inflammatory and microglial response following exposure to variable shockwave pressure-time signatures

4.1. Introduction

With the increased prevalence of blast-induced traumatic brain injury (bTBI) due to changes in enemy tactics, better armor, and increased front-line medical care [166], the lasting side effects of bTBI on service women and men has become a topic of intense interest. Clinical evidence and reports from Operations Enduring Freedom/Iraqi Freedom (OEF/OIF) have provided strong evidence that bTBI results in long-lasting side-effects in those exposed [110]. Symptoms of blast injury that can persist, and even exacerbate, long after the period of canonical secondary damage include apathy, lethargy with psychomotor dystonia and mental blockage, cerebral arterial vasospasm, convulsions, memory impairment, and paralysis [95, 108, 109].

In addition to mechanical damage and its associated secondary pathological events, other investigators have found secondary astrocytic and inflammatory responses to traumatic brain injury [167] including that from blast [96, 123, 154, 168]. Past studies have localized GFAP staining to areas consistent with injury to cortex closest to the blast wave or to areas of gray matter-white matter interface [103, 112, 154, 167]. Serum biomarkers of blast injury have begun to be identified and include GFAP [103, 167] and TNF-α [123].
Astrocytosis and astrocytic infiltration into the area of the injury may contribute to containment of necrotic tissue and subsequently released necrotic cell contents [25] or may contain, and possibly nurtures new neurons in the months following the injury [26]. However, given that chondroitin sulfate proteoglycans (CSPGs) expressed by astrocytes in the glial scar formed around areas of injury are known to be inhibitory to axonal growth, astrocytosis could also contribute to halted axonal regrowth or healing mechanisms in the brain similar to that seen in spinal cord injury [28-35]. Potent cytokine and chemokine responses within the injured cerebral cortex can lead to further disruption of normal brain function and repair [42]. Though the link between microglial activation and brain injury is not fully understood, other researchers have speculated that there may be a functional relationship between microglia and dystrophic axons that extends well beyond the site of primary injury whereby activated microglia exist or migrate beyond the immediate injury area to contribute to more remote axonal dysfunction [169]. Therefore, the importance of astrocytic and microglial responses to injury in both understanding the mechanisms associated with blast-induced brain injury and in developing treatment paradigms that can combat secondary degeneration and facilitate repair cannot be overstated.

We utilized the McMillan Blast Device (MBD, multi-mode shocktube capable of producing shockwaves of unique pressure-time signatures) to determine the influence of positive phase duration and impulse on the amount of astrocytosis and inflammatory response in the brain due to blast. In this study, astrocytic and microglial activation are used as markers of secondary injury pathology associated with exposure to shockwaves. In addition to secondary injury pathology indicative of primary mechanical damage due
to blast in the brain, the contribution of positive phase duration and impulse on the release of detectable serum biomarkers of brain injury following blast injury was examined both to assess the potential for systemic involvement, and to determine the feasibility for non- or less-invasive means of bTBI detection. Additionally, a physiologic response of weight loss in rats following shockwave exposure is reported.
4.2 Materials and Methods

4.2.1 Animal Use

All animal use procedures utilized in this study were performed in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Animals had access to food and water *ad libitum* throughout the course of this study. Adult (9-week; 200-300g), male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN, USA) were separated into individual animal cages, sedated (diazepam, 4mg/kg, i.p.), and transported via climate controlled passenger van from the animal housing facility to the blast site. At the blast site, the animals were maintained in a climate-controlled room with adequate lighting for the duration of their time at the blast site. Immediately prior to injury, animals were deeply anesthetized (ketamine 60mg/kg, xylazine 7.5mg/kg, i.p.), fitted with a Kevlar vest, placed within polyethylene netting (Industrial Netting, Minneapolis, MN, USA), and loaded into the McMillan Blast Device (MBD) [70] in the prone position with their left side facing the shockwave source. Animals were subjected to compressed air- or oxyhydrogen-driven blasts of 175kPa or 250kPa peak overpressure, respectively. Animals were removed immediately following blast in order to prevent confounding effects of hypoxia and acute carbon monoxide exposure [70]. Following blast injury, animals were returned to their individual cages with heat support (Deltaphase Isothermal Pads, Braintree Scientific, Braintree, MA, USA) to prevent anesthesia-induced hypothermia. Animals were maintained in dorsal recumbency for the post-blast anesthesia period and were periodically monitored for respiration. Cardiac activity was monitored via thoracic
palpation. Due to the fact that headache is known to occur in humans exposed to blast, rats were weighed and given carprofen (7mg/kg, i.p.) every 12h after injury until their scheduled euthanasia time. Once recovered from anesthesia, all animals were placed in clean individual cages and transported back to the University of Kentucky animal facility where they remained in their individual cages until euthanasia.

4.2.2 Euthanasia and Tissue Collection

At 3h, 24h, or 72h post injury animals were given an overdose of sodium pentobarbital (150mg/kg, i.p.). Time points were chosen based on known peak expression times of chosen markers following other modes of traumatic brain injury [24, 96, 126]. Once animals were deeply anesthetized, thoracotomy was performed and 1mL of whole blood was collected via syringe from the inferior vena cava. Animals were then transcardially perfused with 200mL of phosphate-buffered saline (PBS), followed by 200mL of 4% paraformaldehyde in PBS. Animals used for Western blot analysis were perfused only with PBS. Brains used for immunohistochemistry were placed in 4% paraformaldehyde in PBS overnight at 4°C, followed by three days in 30% sucrose in PBS at 4°C for cryoprotection, after which they were embedded in OCT compound and stored at -80°C until sectioning. Brains used for Western blot analysis were separated into cerebrum and brainstem/cerebellum sections by severing the cerebral peduncles and brainstem-diencephalon connections. The cerebrum was further subdivided by a midsaggital bisection creating left and right cerebrum samples. The left and right cerebrum samples were further subdivided into dorsal and ventral sections by horizontal
cuts just ventral to each hippocampus. These cuts resulted in four cerebrum samples for each animal: ipsilateral ventral cortex (IVX), ipsilateral dorsal cortex (IDX), contralateral ventral cortex (CVX), and contralateral dorsal cortex (CDX) (See Fig. 3.4 for diagram of sections). Brainstem (BS) and cerebellum (CBM) were separated by severing the cerebellar peduncles. Each sample was wrapped in aluminum foil, flash frozen in powdered dry ice, and kept frozen at -80°C until processing for protein.

4.2.3 Immunohistochemistry

Brains were sectioned at 40µm thickness on a freezing stage sliding microtome (Microm HM 440 E, Microm, Walldorf, Germany). Sections were collected in Tris-buffered Saline (TBS). Four sections from each animal representative of four different anterior-posterior levels (Bregma 0.0, -2.8, -4.8, -6.8) were blocked for 1 hour in 1% horse serum in TBS and treated in primary antibody in 1% horse serum in TBS for 1 hour at room temperature. Antibodies against glial fibrillary acidic protein (GFAP, Mouse anti-GFAP, 1:50,000, Millipore, Billerica, MA, USA) and ionized calcium binding adapter molecule 1 (Iba1, Rabbit anti-Iba1, 1:10,000, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were diluted in TBS with 1% horse serum and applied to the sections overnight at room temperature. Anti-GFAP and anti-Iba1 primary antibodies were detected using the appropriate species-specific secondary antibodies conjugated to biotin (1:5,000, Jackson Immunoresearch). Biotinylated antibody treated slices were treated with streptavidin-conjugated horseradish peroxidase (1:5,000, Jackson Immunoresearch). Presence of antibody reactivity was detected via enzymatic oxidation of 3,3’-
diaminobenzidine (DAB, Jackson Immunoresearch). Following staining, slides were 
mounted on to slides (Fisher SuperFrost Plust, Fisher Scientific, Waltham, MA, USA) 
and mounted with Permount (Fisher Scientific) and coverslips. Slides were imaged on an 
Olympus AX 80 microscope. Iba1 positive cells exhibiting a reactive, rod-shaped 
morphology, defined as having a cell body length at least twice that of its width, were 
counted in the ventral posteriolateral/posteriomedial (VPL) nuclei and in the cortex 
inferior to the entorhinal notch and lateral to the midline of the brain in both sides of the 
brains of rats subjected to sham, oxyhydrogen-driven and compressed air-driven 
shockwaves. These counts were made in all four sections from each animal. Results are 
reported as number of counted cells per area (ipsilateral or contralateral to blast) or whole 
brain per treatment group. GFAP-stained images were examined for patterns of staining 
and are reported descriptively.

4.2.4 Western Blotting

Frozen brain tissue was weighed, covered with 150µl RIPA Buffer (Radio-
immunoprecipitation Assay Buffer) and allowed to thaw on ice. Thawed tissue was 
chopped, placed in a dounce homogenizer with cell lysis buffer (2ml per g of tissue), and 
homogenized by 20 passes in the homogenizer. Resulting lysates were purified by 
centrifugation (2X 15min at 13.2 rcf). Protein concentrations were quantified by 
bicinchoninic acid (BCA) Protein Assay (Thermo Scientific). Lysates were separated by 
SDS-PAGE (10 % Bis-Tris, Invitrogen) using MOPS Running Buffer (GFAP, 
Invitrogen) or MES Running Buffer (Iba1, Invitrogen), transferred to nitrocellulose
membranes via semi-dry protein transfer (Trans-Blot Turbo Transfer System, Bio-Rad, Carlsbad, CA, USA). Membranes were blocked in Tris-buffered Saline (TBS) containing 5% (w/v) non-fat powdered milk (GFAP) or 5% (v/v) normal horse serum (Iba1) for one hour at room temperature to prevent non-specific antibody-protein interaction. Once blocked, membranes were treated with TBS containing 0.05% Tween-20 (TTBS) and primary antibody (GFAP – Millipore; Iba1 – Wako) overnight at 4°C. Membranes examining tissue GFAP content were treated with anti-GFAP antibody and anti-Actin antibody concurrently. Membranes examining tissue Iba1 content were treated first with anti-Actin antibody, imaged, then treated with anti-Iba1 antibody and imaged later. Anti-Membranes were washed 3 times for 5 minutes with TTBS, then treated with TTBS containing the appropriate secondary antibody conjugated to an infrared fluorophore (1:5,000, Rockland). Following secondary antibody treatments, membranes were washed 3 times for 5 minutes with TTBS, then imaged on an Odyssey Scanner (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed for relative band intensity on the Image Studio software (Li-Cor).

4.2.5 Serum Biomarker Evaluation

Blood collected from animals immediately prior to perfusion was allowed to clot for 2 hours on ice, then centrifuged at 4,000 x g for 15 minutes to separate cells and platelets from serum. Serum was snap frozen on dry ice and stored at -80°C until use. Serum GFAP levels were examined by SDS-PAGE separation on 10% Bis-Tris gels (Invitrogen). Proteins were then blotted onto nitrocellulose membranes via semi-dry
protein transfer (Trans-Blot Turbo Transfer System, Bio-Rad, Carlsbad, CA, USA).
Membrane blocking, antibody treatments, and imaging were the same as listed for GFAP
above. GFAP band intensities were compared to those of actin on the same blot and
reported as a ratio. Serum tumor necrosis factor alpha (TNF-α) levels were quantified by
ELISA (R & D Systems) according to the manufacturer’s instructions.

4.2.6 Statistical Analysis

All data were analyzed by ANOVA. The Student Newman-Keuls post hoc
analysis was selected for comparisons between treatment groups due to its ability to
protect against both Type I and Type II statistical error.
4.3 Results

4.3.1 Astrocytosis following shockwave exposure

Evidence of reactive astrocytosis was found in the brains of rats exposed to compressed air-driven shockwaves (Fig. 4.1, asterisks). Increased GFAP staining consistent with reactive astrocytosis was found in the brains of 4 of 6 rats exposed to compressed air-driven shockwaves. The location of increased GFAP staining was also consistent with increased IgG staining (previously described in Chapter 3) and consisted of highly specific labeling of astrocytes with a reactive morphology (Fig. 4.2). Regionally specific increases in GFAP expression were not noted in sham- or oxyhydrogen-exposed rat brains (Fig. 4.1). Increased GFAP protein levels were not detected by Western blot (Appendix E).
Fig. 4.1. Regionally specific, bilaterally asymmetric astrocytosis, in compressed air-exposed rat brains. Increased GFAP expression was present in the IVX of the brains of rats exposed to compressed air-driven shockwaves (asterisk). Note the lack of regionally specific or bilaterally asymmetric GFAP staining in sham- or oxyhydrogen-driven shockwave-exposed rat brains. n=6 per treatment group.
Fig. 4.2. Astrocytic morphology changes following compressed air-driven shockwave exposure. Compressed air-driven shockwaves resulted in increased GFAP staining and astrocytic morphology changes consistent with a reactive phenotype in rat brain. Note astrocytic end-feet placement on blood vessels (black arrows). These changes were not
present in sham-injured animals (top panels). Brains of oxyhydrogen-driven shockwave exposed animals were similar to sham-injured brains and were omitted for brevity. Compressed air-injured animal images representative of 4 of 6 animals injured. Sham-injured animal images representative of all sham-injured animals (n=6 per treatment group).
4.3.2 Microglial morphology changes in response to compressed air-driven shockwaves.

Changes in microglial morphology were present in the cortex and thalamus of rats exposed to compressed air- and oxyhydrogen-driven shockwaves. Microglia staining positive for ionized calcium binding adapter molecule-1 (Iba1) and that had a cell body longitudinal axis equal to or greater than twice their width were present in sections from compressed air- and oxyhydrogen-injured animals (Fig. 4.4), however the density of rod-shaped microglia in compressed air-injured animals was significantly higher than in oxyhydrogen- or sham-injured animals. Microglia morphology was limited to normal resting or rod-shaped morphology only and there were no microglia with a classically activated morphology noted. Rod-shaped microglia were present throughout the rostral-caudal extent of the piriform and surrounding cortex, however they were most dense in the sections with fully developed hippocampi (R-C levels 2 and 3, Fig. 4.5).
Fig. 4.3. Microglial morphology changes following blast injury. Iba1 stained brain sections of rats subjected to sham injury showed no microglial morphology changes in the piriform cortex (A, C-D) and ventral posteriolateral nucleus (VPL, B, E-F) of the
thalamus. Iba1 stained sections from the brains of rats subjected to blast injury showed microglial morphology changes consistent with rod-shaped microglia formation in the piriform cortex (G, I-J) and VPL (H, K-L). Note the classic orientation of rod-shaped microglial longitudinal axis which lines up perpendicular to cortical surface when present in cortex and parallel to thalamic surface when present in the VPL. Panels G & H are only representative of compressed air-injured animals in terms of rod-shaped microglia density, however the morphology of individual rod-shaped microglia in oxyhydrogen-injured animals is identical to that presented in panels I-L when present. Images representative of 6 animals in each treatment group.
4.3.3 Rod-shaped microglia formation in response to compressed air-driven shockwaves

Rats exposed to compressed air-driven shockwaves exhibited statistically higher numbers of rod-shaped Iba1-positive microglia in the ipsilateral and contralateral piriform cortex (IPX/CPX; Fig. 4.4 B & C) and in their ipsilateral and contralateral ventral posteriolateral nuclei (IVPL/CVPL; Fig. 4.4 D & E). Rod-shaped microglia development followed a time course typical to amoeboid activated microglia formation with some activated rod-shaped microglia being detected at 3 hours post injury, more detectable at 24 hours post injury, and the highest number of activated rod-shaped microglia detectable at 72 hours post injury in the whole brain (Fig. 4.4 A). This same developmental time course was displayed in each anatomical region in which rod-shaped microglia were found (Fig. 4.4 B-E).
Fig. 4.4. Rod-shaped microglia density by anatomical region and time course of development. Rod-shaped microglia were detected in animals injured by both oxyhydrogen- and compressed air-driven shockwaves, however only those animals injured by compressed air-driven shockwaves exhibited statistically higher numbers of rod-shaped microglia (A). Statistically higher numbers of rod-shaped microglia were present in the ipsilateral piriform cortex (IPX, B), contralateral piriform cortex (CPX, C), ipsilateral ventral posteriolateral (IVPL, D) and contralateral ventral posteriolateral (CVPL, E) nuclei of the thalamus of compressed air-injured animals. The time course of rod-shaped microglia development followed a classic profile with a few rod-shaped microglia being detectable at 3h, more detectable at 24h, and the most being detected at 72h in every anatomical region examined (B-E) and in the brain as a whole (A). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, error bars +SD, n=6 per treatment group.
4.3.4. Anterior-posterior distribution of rod-shaped microglia formation in response to compressed air-driven shockwaves

Rod-shaped microglia were found in the entire anterior-posterior distribution of the ipsilateral and contralateral piriform cortex of shockwave-exposed rats, however there were statistically more rod-shaped microglia in these regions in the brains of compressed air-exposed rats than oxyhydrogen-exposed rats (Fig. 4.5 A & B). Sporadic rod-shaped microglia were found throughout the dorsolateral thalami of compressed air-injured animals, however statistically higher levels were found in the ventral posteriolateral nuclei of compressed air-injured animals compared to sham- and oxyhydrogen-injured animals (Fig. 4.5 C & D).
Fig. 4.5. Anterior-posterior rod-shaped microglia distribution. Rod-shaped microglia were found in the entire anterior-posterior distribution of the piriform cortex on both the ipsilateral (IPX; blast side) and contralateral (CPX; side opposite blast) sides of the brain (A-B). Statistically higher numbers of rod-shaped microglia were found in the middle two sampled piriform cortices of compressed air-injured rat brains compared to sham- and oxyhydrogen-injured rat brains (A-B). Though sporadic rod-shaped microglia were present in the entire dorsolateral thalamus of compressed air-injured animals, they were most concentrated in the ventral posteriolateral nuclei of the ipsilateral and contralateral (IVPL and CVPL, respectively) sides of the brains of rats exposed to compressed air-driven shockwaves. Green stars represent p-values comparing compressed air-injured rats to sham. Purple stars represent p-values comparing compressed air-injured rats to oxyhydrogen-injured rats. One-way ANOVA, error bars +SD, **p<0.01, ***p<0.001, ****p<0.0001, n=6 per treatment group.
4.3.5 Serum GFAP levels in response to blast

Serum levels of glial fibrillary acidic protein (GFAP) were not elevated in response to either oxyhydrogen- or compressed air-driven shockwave exposure at 3h, 24h, or 72h post injury (Fig. 4.6).
Fig. 4.6 – Serum GFAP levels in response to blast. Western blot analysis of glial fibrillary acidic protein (GFAP) levels did not reveal any significant changes in response to blast. One-way ANOVA, error bars +SD, n=6 per treatment group.
4.3.6 Serum TNF-α levels in response to blast

Serum levels of tumor necrosis factor alpha (TNF-α) were significantly elevated in both compressed air- and oxyhydrogen-driven shockwave-exposed animals at 72 hours post injury, but not at 3h or 24h post injury (Fig. 4.7).
Fig. 4.7 – Serum TNF-α levels in response to blast. Analysis of serum levels of tumor necrosis factor alpha (TNF-α) by ELISA revealed elevated levels at 72h post injury in both oxyhydrogen- and compressed air-injured animals. One-way ANOVA, error bars +SD, ***p<0.001, n=6 per treatment group.
4.3.7 Weight loss following blast injury

Following blast injury, rat weights dropped over the course of 60 hours following shockwave exposure (Fig. 4.8). Significant weight loss was detected by 48 hours post injury which persisted through 60 hours post injury (Fig. 4.8). There was no statistically significant difference in weight loss between shockwave sources.
Fig. 4.8 – Rat weight loss following blast injury. Analysis of rat weights at 12-hour intervals following shockwave exposure revealed significant weight loss by 48 hours that persisted through 60 hours. One-way repeated measures ANOVA within an individual blast source exposure group, *$p<0.05$, **$p<0.01$ compared to base weight percentage, error bars $\pm$SD, $n=6$ per treatment group.
4.4 Discussion

We have presented secondary effects of shockwave exposure that last up to three days after the injury. Astrocytic response following shockwave exposure was present in the same region or bordering the region where IgG extravasation was at its greatest as evidenced by increased GFAP staining intensity around the ipsilateral ventral cortex, hypothalamus, and borders of the internal capsule. Additionally, astrocytic morphological changes consistent with a reactive phenotype were noted on the borders of the internal capsule. Interestingly, glial scar and microglial activity has been found on the edges of the internal and external capsules following lateral fluid percussion injury [25]. Lateral fluid percussion injury is a semi-diffuse model of concussive brain injury which shares many pathological characteristics with bTBI. Taken together, these results suggest secondary injury pathology associated with areas of tissue-density difference which are areas predicted to experience significant shear stress in blast injury [170]. While some degree of hematoma was found on the ventral surface of the brains of rats exposed to both modes of the MBD (Chapter 3), focal astrocytosis and microglial activation were virtually exclusive to compressed air-driven shockwave exposure suggesting positive phase duration may play a particularly important role in the long-term secondary injury cascade following blast exposure.

Glial scar formation has been shown to be detrimental to rebuilding CNS axonal structure following traumatic injury [28-34]. Though we have not presented evidence of glial scar formation in our model, the astrocytic response reported in the present study is consistent with that reported by others to result in eventual glial scar formation [171].
Given the inhibitory effect of CSPGs produced by the astrocytes in the glial scar, and the intensity and location of GFAP staining presented here, the astrocytic response seen in compressed air-driven shockwave-exposed rats may lead to widespread sensory/motor deficits following blast exposure. In contrast, increased positive phase duration leading to astrocytic changes may result in aberrant sprouting leading to inappropriate integration of sensory information [26]. Communication, pain, sensory integration, and movement disorders that develop weeks to months after injury have all been linked to blast exposure [95, 108-110].

Astrocytes play a pivotal role in the formation of the blood-brain barrier by ferrying nutrients and waste products between neurons and other glia and the vascular supply. Increased GFAP protein expression was visualized in the area bordering increased IgG extravasation and was unsurprising given the close interrelationship between astrocytes and blood-brain barrier integrity. Additionally, astrocytic activation has been shown to occur following exposure to blood-borne cytokines that are not normally present in the brain parenchyma [172], providing a possible mechanism for astrocytic activation in compressed air-injured animals from the present study. Indeed, astrocyte swelling in response to high extracellular potassium from broad neuronal excitation and BBB disruption following TBI has been reported [173, 174]. In addition, astrocytes are active participants in neurotransmission through their interaction in the tripartite synapse where their role in neurotransmitter clearance and calcium signaling is crucial to normal neuron-neuron communication [175]. Changes in astrocytic morphology and behavior of the magnitude observed in the present study could potentially lead to serious deficits in brain physiology.
The immune response has been studied extensively as a pathological outcome in both diffuse and focal models of traumatic brain injury [36, 115-119]. Although classical amoeboid activated microglial morphology has been reported in other models of blast-induced brain injury, only rod-shaped cells or resting microglia were present in the piriform cortices and in the ventral posteriolarateral nuclei (VPL) of shockwave-exposed rats. Additionally, significantly higher numbers of rod-shaped microglia were present in these areas in compressed air-injured rats than in sham- or oxyhydrogen-injured rats, indicating a differential amount of microglial activation in response to different shockwave sources. Rod-microglia are thought to be involved in stripping of synapses [120], and have been observed in reports of animal models of traumatic CNS insults [121, 122]. The piriform cortex is highly developed in the rat and is involved in olfactory processing as well as memory integration while the VPL is the thalamic relay nucleus for ascending sensory information from the body to the sensory cortex. Clinical symptoms such as impaired memory function and psychomotor dystonia could be due, at least partially, to damage in the piriform cortex and VPL. Interestingly, while rats exposed to compressed air-driven shockwaves exhibited bilateral asymmetry with respect to blood-brain barrier compromise (Chapter 3) and astrocytosis, there was no significant asymmetry to rod-shaped microglia formation, indicating secondary pathology may be more widespread than previously thought [24, 96, 126].

Serum biomarkers of traumatic brain injury could provide a less-intrusive, early detection screening mechanism for detection of blast-induced brain injury [17]. Of all populations which could benefit from such biomarkers, the military perhaps stands to benefit the most due to the rapid availability of front-line medical care in the event of a
detected brain injury [68, 165, 166, 168]. Serum GFAP and TNF-α have been reported as elevated from 6 hours to 7 days (GFAP) and at 3 to 48 hours (TNF-α) following bTBI [103, 123] and in conventional TBI mechanisms as well [167]. While localized GFAP levels were elevated in the same region as blood-brain barrier compromise, serum GFAP levels were not elevated in shockwave-exposed rats at 3h, 24h, or 72h following injury suggesting that GFAP was upregulated in astrocytes due to the injury but that the astrocytic membranes remained intact and could release GFAP into systemic circulation. TNF-α levels were elevated at 72h post injury in shockwave-exposed rats and there was statistically no difference between shockwave-exposed groups at any time point tested. TNF-α has been reported to be increased in brain tissue following blast injury [123], however the present study is the first report of elevated serum TNF-α following blast injury. Elevated serum TNF-α could have been detected after systemic accumulation of TNF-α released from brain tissue, however it could also be due to a mounting systemic inflammatory response due to contusion following whole-body blast exposure [176, 177]. Though we have shown a differential response in histopathological outcome measures in the brain with respect to shockwave source, markers of inflammation are detectable in peripheral serum without exposure to parameters leading to detectable BBB compromise, astrocytosis, or microgliosis as evidenced by increased serum TNF-α levels in oxyhydrogen-driven shockwave-exposed animal.

Though not reported in case studies or retrospective human blast-injury studies to-date, weight loss has been reported in human case studies of other TBI mechanisms secondary to loss of smell and taste or through neuroendocrine changes brought on by pituitary damage/dysfunction [178] and in mice following bTBI [179]. Although stress
brought on by transport, handling, or indirect shockwave sound exposure cannot be ruled out as causes for weight loss in the shockwave-exposed rats in this study due to the lack of time-matched sham-exposed animals, weight loss could also be a side-effect of systemic mediators of the inflammatory response (such as TNF-α) [180, 181] or directly from brain injury [178].

Individuals have been injured by blasts in both open air and enclosed spaces; however those placed between the blast and a wall often experience injuries between two to three times as severe as those equally positioned from the source, but not near the building [68]. Absent secondary (shrapnel) or tertiary (acceleration/deceleration of the body) injuries, these reports of increased injury may be due to elongated positive phase durations due to reflected blast waves from the structure. Our data support the hypothesis that increased positive phase duration can lead to increased blast-associated pathology. The localization of BBB damage and astrocytic changes indicative of injurious exacerbation to the ipsilateral ventral cortex, thalamus, and hypothalamus could contribute to memory and sensory abnormalities classically seen as part of human bTBI case reports [182, 183]. Clinical studies comparing injuries sustained by individuals exposed to open-air blasts to those sustained by individuals exposed to blasts while in an enclosure would be difficult due to the variability of individual environments and explosive charge volume. However, based on the results of the studies outlined in this chapter and chapter 3, we would expect those individuals exposed to shockwaves within an enclosure or between the explosive and a solid structure to exhibit more immediate and long-term symptoms of blast-induced brain injury than those exposed to the same blast at the same distance not compounded by the enclosure or structure. Though the
increased markers of brain pathology associated with longer positive phase durations exhibited in this study could signify potentially long-lasting behavioral, emotional, or sensory/motor abnormalities, there may also be additional facets of the blast injury shared by animals injured in either mode of the MBD that were not detected in our histopathological evaluation, but could be associated with increased serum TNF-α levels or weight loss.

This study represents the first time differential secondary injury cascades have been reported following shockwave injury from two different sources within the same injury device. Additionally, this is the first report of rod-shaped microglial formation and of increased serum levels of TNF-α levels following blast injury, two important events in the inflammatory process resulting from TBI. Together, these studies shed light on the cause and profile of the secondary injury cascade following blast-induced traumatic brain injury which can aid in future diagnosis, decision-making regarding future treatment paradigms, and design of blast-wave mitigation technologies.
5. CHAPTER 5

Blast-induced head rotation in the rat is not responsible for injury differences between shockwave sources

5.1 Introduction

Though strong evidence exists for the existence of a brain injury due to blast obtained from exposure to the shockwave alone, secondary, tertiary and quaternary mechanisms of blast injury can create complex, polytrauma scenarios for those exposed to blast as well as produce confounding data for laboratory models of blast-induced brain injury [184]. Secondary blast injury from projectiles put into motion by the blast event would produce injuries similar to traditional TBI mechanisms of closed/open head or penetrating head injury. Tertiary mechanisms where all or part of an individual is put into motion by the blast wave could also result in traditional TBI injury due to acceleration-deceleration events and rapid mechanical loading.

Head rotation has been identified as a major source of diffuse brain injury [185]. Mechanical stress and shearing mechanisms due to rapid acceleration/deceleration of the brain are known causes of primary brain injury as well as initiators of secondary injury pathology [186]. Defining features of rotational injury include diffuse axonal injury, coma and diffuse swelling [57, 106, 187-190]. Rotational injury due to blast has been shown to result in blood-brain barrier disruption in a mouse model of blast-induced brain injury [125].
We developed and characterized a rat model of blast injury that utilizes a multi-mode shock tube (McMillan Blast Device; MBD) to produce shockwaves with different unique pressure-time signatures [70]. We analyzed the head movement of rats subjected to each mode (compressed air or oxyhydrogen) of the MBD for differences in head movement angle or velocity between each mode and compared extravasated IgG and GFAP in the ipsilateral ventral cortex of shockwave-exposed animals to head rotation angle and velocity. Though oxyhydrogen-driven shockwaves exhibited a higher peak overpressure than the compressed air-driven shockwaves, the impulse (and, thus, energy) delivered by a compressed air-driven shockwave was greater than that of an oxyhydrogen-driven shockwave. This study introduces the first analysis of head movement following blast injury in a rat model. Furthermore, we demonstrate a differential brain injury resulting from shockwaves of different pressure-time signatures that appears to be independent of the degree of head movement following blast. The results of this study provide support for the importance of the positive phase duration on blast-induced brain injury as well as highlight a possible confounding factor that must be taken into account in blast injury research.
5.2 Materials and Methods

5.2.1 Animal Use

All animal use procedures utilized in this study were performed in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Animals had access to food and water *ad libitum* throughout the course of this study. Adult (8-week), male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN, USA) were separated into individual animal cages, sedated (diazepam, 4mg/kg, i.p.), and transported via climate controlled passenger van from the animal housing facility to the blast site. At the blast site, the animals were maintained in a climate-controlled room with adequate lighting for the duration of their time at the blast site. Immediately prior to injury, animals were deeply anesthetized (ketamine 60mg/kg, xylazine 7.5mg/kg, i.p.), fitted with a Kevlar vest, placed within polyethylene netting (Industrial Netting, Minneapolis, MN, USA), and loaded into the McMillan Blast Device (MBD) [70] in the prone position with their left side facing the shockwave source. Animals were subjected to compressed air- or oxyhydrogen-driven blasts of 175kPa or 250kPa peak overpressure, respectively. Animals were removed immediately following blast in order to prevent confounding effects of hypoxia and acute carbon monoxide exposure [70]. Following blast injury, animals were returned to their individual cages with heat support (Deltaphase Isothermal Pads, Braintree Scientific, Braintree, MA, USA) to prevent anesthesia-induced hypothermia. Animals were maintained in dorsal recumbency for the post-blast anesthesia period and were periodically monitored for respiration and cardiac activity was monitored via thoracic palpation. Due to the fact that
headache is known to occur in humans exposed to blast, rats were weighed and given carprofen (7mg/kg, i.p.) every 12h after injury until their scheduled euthanasia time. Once recovered from anesthesia, all animals were placed in clean individual cages and transported back to the University of Kentucky animal facility where they remained in their individual cages until euthanasia.

5.2.2 Head Rotation Analysis

A three-inch porthole was cut into the MBD and was replaced with Plexiglas. Rat head movement during the injury was recorded via high-speed video camera (300 frames-per-second; Sanyo Xacti HD 1010). A camera was mounted on the external surface of the MBD with the lens 2.5 inches above the window. An alternative light source similar to that used with a surgery scope was used to illuminate the rat head during the injury through the Plexiglas window. Videos were scanned by an observer who was blind to the injury parameters for the three specific events: the final frame before the shockwave reaches the rat (Fig. 5.1A), the frame depicting maximal head deflection, and the frame depicting maximal head reflection. A composite image was made containing the head in each of the three positions was made and a line was drawn down the midline of each of the three heads in the picture (Fig. 5.1B). The intersect of the three lines was used to determine the angle of maximal deflection and the angle of maximal reflection. The distance between the nose on each of the three heads in the picture was used to determine the maximal distance of deflection and reflection and the number of frames each rat’s head took to reach the deflection or reflection position was divided by 300 to determine
the time of head travel in milliseconds. Distance of deflection or reflection was combined with the time of head travel to determine the average velocity of the head in each direction.
Fig. 5.1. Determination of head movement angles during blast exposure. The last frame before movement of the rat head was identified on high speed video of the blast injury event (A). A line was drawn down the middle of the head and the midpoint between the eyes was identified (black star) for each of three frames: initial head position (position A; blue-green line), position of maximal deflection (position B; red-green line) and position of maximal head rebound (position C; yellow-green line) (B). The angles between positions A and B, and between positions B and C were measured (B). The number of frames between each position was also noted as a measure of time (not shown). Representative images shown.
5.2.3 Euthansia and Tissue Collection

At 3h, 24h, or 72h post injury animals were given an overdose of sodium pentobarbital (150mg/kg, i.p.). Time points were chosen based on known peak expression times of chosen markers following other modes of traumatic brain injury [24, 96, 126]. Once animals were deeply anesthetized, then transcardially perfused with 200mL of phosphate-buffered saline (PBS). After perfusion, animal brains were removed and photographed for evidence of hematoma and petechial hemorrhage. Brains were separated into cerebrum and brainstem/cerebellum sections by severing the cerebral peduncles and brainstem-diencephalon connections. The cerebrum was further subdivided by a midsagittal bisection creating left and right cerebrum samples. The left and right cerebrum samples were further subdivided into dorsal and ventral sections by horizontal cuts just ventral to each hippocampus. These cuts resulted in four cerebrum samples for each animal: ipsilateral ventral cortex (IVX), ipsilateral dorsal cortex (IDX), contralateral ventral cortex (CVX), and contralateral dorsal cortex (CDX) (See Fig. 3.4B for diagram of sections). Brainstem (BS) and cerebellum (CBM) were separated by severing the cerebellar peduncles. Each sample was wrapped in aluminum foil, flash frozen in powdered dry ice, and kept frozen at -80°C until processing for protein.

5.2.4 Western Blotting

Frozen brain tissue was weighed, covered with 150µl RIPA Buffer (Radio-immunoprecipitation Assay Buffer) and allowed to thaw on ice. Thawed tissue was chopped, placed in a dounce homogenizer with cell lysis buffer (2ml per g of tissue), and
homozenized by 20 passes in the homogenizer. Resulting lysates were purified by centrifugation (2X 15min at 13.2 rcf). Protein concentrations were quantified by bicinchoninic acid (BCA) Protein Assay (Thermo Scientific). Lysates were separated by SDS-PAGE (10 % Bis-Tris, Invitrogen) using MOPS Running Buffer (Invitrogen), transferred to nitrocellulose membranes via semi-dry protein transfer (Trans-Blot Turbo Transfer System, Bio-Rad, Carlsbad, CA, USA). Membranes were blocked in Tris-buffered Saline (TBS) containing 5% (w/v) non-fat powdered milk for one hour at room temperature to prevent non-specific antibody-protein interaction. Once blocked, membranes were treated with TBS containing 0.05% Tween-20 (TTBS) and primary antibody (anti-IgG; 1:1,000, Sigma) overnight at 4°C. Membranes were washed 3 times for 5 minutes with TTBS, then treated with TTBS containing the appropriate secondary antibody conjugated to an infrared fluorphore (1:5,000, Rockland). Following secondary antibody treatments, membranes were washed 3 times for 5 minutes with TTBS, then imaged on an Odyssey Scanner (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed for relative band intensity on the Image Studio software (Li-Cor).

5.2.5 Statistical Analysis

All data were analyzed by One- or Two-Way ANOVA. The Student Newman-Keuls post hoc analysis was selected for comparisons between treatment groups due to its ability to protect against both Type I and Type II statistical error. Correlation analyses were performed when comparing pressure, impulse, IVX IgG or GFAP content to head rotation angle or velocity.
5.3 Results

5.3.1 Blast-induced Head Rotation

Head rotation due to blast was examined via high-speed (300 f.p.s.) video camera. Though there are significant differences between oxyhydrogen and compressed air in both the velocity of shockwaves (Fig. 2.2) and the amount of kinetic energy contained within the shockwaves (Fig. 2.8), there was no relationship between the peak overpressure or the impulse to which the rats were exposed and the maximal angle of head deflection/rebound (Fig. 5.2) or the maximal velocity of head deflection/rebound (Fig. 5.3). Additionally, there was no significant difference in the total angle through which the head moved (Fig. 5.4) or in the average velocity of head movement (Fig. 5.5) between shockwave sources. The angle and velocity of head rotation failed to correlate with IVX IgG extravasation and GFAP content as measured by Western blot (data not shown). Head movement was not completely lateral and included some rotation about the longitudinal axis of the rats body, thus exposing the inferior surface of the rat’s skull to slightly more blast wind at the time point of maximal deflection.
Fig. 5.2. Angle of blast-induced head movements. The maximal head deflection (orange) or rebound (green) was not significantly affected by the peak pressure (A) or impulse (B) to which the rats were exposed. n=15 per treatment group.
Fig. 5.3. Velocity of blast-induced head movements. The maximal head deflection velocity (orange) or rebound velocity (green) was not significantly affected by the peak pressure (A) or impulse (B) to which the rats were exposed. n=15 per treatment group.
Fig. 5.4. Maximal angle of head deflection and rebound following shockwave exposure from different sources. The higher pressure of oxyhydrogen-driven shockwaves did not result in greater maximal head deflection or rebound (A & B). Increased impulse present in compressed air-driven shockwaves did not contribute to greater total head deflection or rebound (C & D). Shockwave source did not contribute to the maximal angle of head deflection (A & C) or rebound (B & D). n=17-19 per treatment group.
Fig. 5.5. Head deflection and rebound velocity following shockwave exposure from different sources. The higher pressure of oxyhydrogen-driven shockwaves did not result in faster head deflection or rebound velocity (A & B). Increased impulse present in compressed air-driven shockwaves did not contribute to faster head deflection or rebound velocity (C & D). Shockwave source did not contribute to the velocity of head deflection (A & C) or rebound (B & D). n=17-19 per treatment group.
5.4 Discussion

IgG staining in previous studies of blast-induced brain injury was centered around the ventricles and on the blast and anti-blast sides of the cortex, similar to a coup-contrecoup injury [24, 96]. In the present study, observational analysis of head rotation following blast revealed a slightly uneven exposure of the rat skull to blast wind at the time point of maximal deflection which could account for the IVX-specific regional localization of IgG staining intensity shown in Figure 3.3. In each case, the rat head rotated about the longitudinal axis of the spinal column to a point where the ventral portions of the head were slightly more exposed to venting gasses than the dorsal aspects of the head. Interestingly, these other models of blast injury utilized rigid animal holding mechanisms that could have contributed to the brain injury through tertiary means [24, 164] by the whole rat or rat head impacting the rigid structure meant to hold them. Given the discrepancy between the IgG staining distribution between the present study and previous studies [24, 164], this may represent a unique incidence of primary blast-induced blood-brain barrier compromise devoid of secondary (projectiles set into motion by the blast wave) or tertiary (abrupt acceleration/deceleration events from body or head motion caused by the blast wave) injury influences.

Maximal head rotation and maximal rotational velocities did not differ as a function of the peak amount of pressure or total impulse to which the animals were exposed. Additionally, maximal head rotation and maximal rotational velocities did not differ between compressed air- and oxyhydrogen-driven shockwave-exposed animals, indicating the differences in cerebrovascular response to blast we report here are not due
to additional or faster head rotation produced by the compressed air-driven mode of the MBD. Head rotation failed to correlate with IgG extravasation or GFAP expression. These results are in direct opposition to a recent study in which head rotation was found to be the major determinant in a mouse model of blast injury [125]. In their recent study, Goldstein and colleagues found that long-term behavioral deficits produced by blast overpressure injury were ameliorated by immobilizing the mouse head during the injury.

Head movement undoubtedly has the potential to play a role in blast injury, however there are conflicting reports on the importance of head movement using different blast injury mechanisms [103, 125] including the studies outlined in this dissertation. The data of Goldstein et al. suggests that head rotation plays important roles in behavioral aspects following injury [103, 125] which may exist without overt immunohistochemical or anatomical pathology. Using Western blotting techniques, we were unable to detect changes in pathological markers of brain injury that correlated with head rotation. Very few reports examining the role of head rotation on blast-induced brain injury exist. Rapid mechanical loading from the blast wave or following wind has the potential to injure the brain and from the discrepancies present in the literature and the data in this dissertation it is clear a detailed examination of the effects of head rotation on the pathology seen after blast is needed.
6. CHAPTER 6

Discussion and Concluding Remarks

6.1 General Observations On Blast Injury In the McMillan Blast Device

Blast-induced traumatic brain injury is a significant problem accounting for up to 60% of combat casualties [77] and affecting up to 15% of infantry soldiers returning from Iraq [80, 85].

We have shown that the positive phase duration of shockwaves produced by the industry standard compressed air-driven membrane rupture differ substantially from those produced by chemical explosives in terms of their positive phase durations when measured within the same device (Fig. 2.5) and from shockwaves of the same peak pressure (Fig. 2.4), which causes compressed air-driven shockwaves to carry a greater impulse than their chemical explosive-driven counterparts (Fig. 2.6). If shockwave measurements are taken at the same distance from the source and within the same device, greater impulse is directly related to greater energy carried by the shockwave [191]. Therefore, compressed air-driven shockwaves carry greater energy than their peak pressure-matched chemical explosive-driven counterparts. This increased energy has the potential to cause more damage to structures, armor, and tissue. Conversely, there is a time frame over which a great deal of energy could be delivered, but less actual tissue damage could occur. This is the same reasoning behind helmet padding or seat belts. In this case, delivering a lower amount of energy but over a shorter period of time could be more detrimental and increased positive phase duration could result in less injury.
Nevertheless, our data suggest that rapid blast events occur within the time range where longer duration at equal pressure leads to more damage.

Rapid mechanical loading due to the interaction of a high frequency stress wave with a low frequency shear wave is thought to be the mechanism by which blast injures tissues of different densities [192]. The interaction of these two waves is greatest at the interface of tissues of different densities, such as the brain-fluid interface and even the gray/white matter interface. In the studies we present in this dissertation, compressed air-driven shockwaves result in increased IgG staining that is centered around the ipsilateral gray-white matter interface of the internal capsule. Additionally, GFAP staining appears to be specific to these borders as well. Rod-shaped microglia were found near the surface of the gray matter in the inferior cortex (piriform cortex) and the nuclei/internal medullary lamina interface surrounding the VPL of the thalamus. The anatomical locations where these markers of injuries were found in this study fit with the hypothesis that stress/shear wave interactions occur at the interface of tissues of different densities. The floor of the rat cranial vault contains ridged bone structures which are in close juxtaposition to the brain matter sitting just over them. Additionally, the ventral surface of the brain does not fit closely into the middle temporal fossa where large amounts of cerebrospinal fluid (CSF) are positioned. Both the large bony structures composing the floor of the cranial vault and the relatively larger volumes of CSF in direct contact with brain matter create prime anatomical locations for high shear/stress wave interaction.

It is difficult to assess which clinical features of TBI following blast exposure are due directly to blast, due to the fact that blasts are often polytrauma situations [68, 77].
Short-term clinical findings of primary blast injuries include apnea followed by rapid breathing, bradycardia, and hypotension [193]. Parasympathetic vagal stimulation resulting from a pulmonary vagal reflex and subsequent cardiovascular decompressor Bezold-Jarish reflex leads to decreased heart rate and dilation of the peripheral blood vessels following blast exposure [194] which could further complicate hypoxic stress from apnea. Assessment of physiologic features of blast injury was difficult in the present study due to the effects of ketamine anesthesia. Some animals injured as part of the present study but subsequently excluded from the results presented here exhibited permanent apnea following blast exposure, however it is difficult to determine the direct cause of apnea which could be due to the effects of the shockwave directly, secondary injury to the respiratory centers in the brainstem and spinal cord, or due to the depressive effects of anesthesia combined with increased parasympathetic vagal response.

Cerebrovascular compromise, including hemorrhage, is also a well-documented effect of blast injury [77, 195-197]. Hematoma secondary to hemorrhage surrounding the median eminence was evident in the brains of rats exposed to both compressed air- and oxyhydrogen-driven shockwaves, however hematoma size and petechial hemorrhage was more extensive in compressed air-exposed rats. The ventral surface of both rat and human brain is the site of convergence for several delicate veins returning blood to the body. These veins converge on the cavernous sinus which is enclosed in dura mater and tightly tied to bone comprising the floor of the cranial vault. Due to the anatomical structure of venous supply in this area, a natural susceptibility in this area for tearing of some of these veins exists which could lead to delayed loss of consciousness or death in
humans with a similar injury. Hematoma associated with epidural or subarachnoid blood vessel damage has also been found in soldiers exposed to blast [77].

Other long-term clinical consequences of blast-induced brain injury include memory loss and irritability, symptoms which can develop immediately following the injury or can follow a protracted developmental time course [77]. In fact, many soldiers do not know they are injured until months or weeks later [77]. Chronic inflammation associated with injury has been implicated in long-term development of TBI symptoms and can result in progression of symptoms long after the initial injury [125]. Given the location and prevalence of rod-shaped microglia in compressed air-exposed animals in the piriform cortex, rod-microglia synaptic restructuring could contribute to changes in memory and sensory association following shockwave exposure. Headache and chronic pain syndrome has been well documented in TBI cases, including blast-induced brain injury [198-201]. Rod-shaped microglia in the thalamic relay center for sensation from the body and face could also be at least partially involved in the development of chronic pain or thalamic pain syndrome in those exposed to blast through synaptic reorganization of general sense or pain relays.

Serum biomarkers of brain injury have been reported and include GFAP [103,167] and TNF-α [123]. We failed to show changes in GFAP levels in either mode of blast injury, however TNF-α levels were elevated in both compressed air- and oxyhydrogen-exposed animals at 72h post injury. Elevated TNF-α levels at 72h would not be useful as a positive sign of blast-induced brain injury for the purposes of decision making regarding post-injury medications to reduce secondary injury due to the lack of
intervention efficacy after approximately 8 hours following injury. However, it may be useful as a future indicator in such instances should treatments that are effective if given later in the course of the injury are developed. Additionally, elevated serum TNF-α levels in the absence of overt behavioral signs of blast injury may be a useful indicator of whether or not a soldier should go back to duty, or perhaps be placed on light duty until their injury has a chance to stabilize.

Head rotation has been shown to cause sufficient rapid mechanical loading to produce brain injury [185]. Rapid acceleration/deceleration of the brain are known causes of primary brain injury and are often initiators of secondary injury pathology [186]. Rotational injury has been linked to diffuse swelling in comatose patients which is most likely secondary to diffuse axonal injury and vasogenic edema [57, 106, 187-190], and rotational injury in blast has been shown to coincide with blood-brain barrier disruption in a mouse model of bTBI [125]. In contrast, our data found no correlation between the amount or velocity of head rotation experienced by rats subjected to blast injury and the amount of IgG extravasation found in those rats. Moreover, head rotation was not found to coincide with reactive astrogliosis or microgliosis in the ipsilateral ventral cortex of blast-exposed rats, suggesting that, while head rotation may play a role in the pathophysiology of blast-induced brain injury, it is clear that the markers of brain injury examined in these studies were stimulated, at least partially, by mechanisms other than head rotation.
6.2 The Effect of Positive Phase Duration on the Injury

Differences in shockwave source between large and small animal models [24, 96, 124, 126] make it difficult to directly compare the pathology observed in each. Our data support the idea that different shockwave sources may produce different injuries and that, in addition to the peak pressure of a shockwave, the positive phase duration is almost certainly important to the brain injury produced. Compressed air-driven shockwaves, which carry more kinetic energy than their chemical explosive-driven counterparts, result in more hematoma, more extensive blood-brain barrier compromise and reactive astrocytosis within and around the injury area, and higher numbers of rod-shaped microglia in anatomical locations which are related to behavioral findings in the clinical blast injury literature.

Interestingly, though the markers of brain injury we examined were non-existent or minimally positive in oxyhydrogen-exposed animals over sham-exposed animals, TNF-α levels were elevated at 72h in both oxyhydrogen- and compressed air-exposed animals, thus indicating there may be some effects of oxyhydrogen-driven shockwave exposure which were undetected. This undetected component of injury responsible for increased serum TNF-α may be produced by head rotation, as head rotation angle and velocity were also found to be not different between oxyhydrogen- and compressed air-injured animals, but has been reported to correlate with blast injury pathology by others [125]. There are other components of a chemical explosive-driven blast which are not accurately modeled by compressed air-driven membrane rupture (i.e., heat, acoustic, electromagnetic) which could contribute to TBI.

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6.3 Future Directions

Though we have demonstrated clear differences in pathology associated with shockwaves of different sources (and, thus, different positive phase durations) in the data outlined in this dissertation, a systematic examination of shockwave duration on injury is still needed. It is possible to vary the positive phase duration of shockwaves from an individual source within a shock tube device. The length of the pressurized, driver chamber can be shortened to reduce the total amount of driver gas needed to rupture the Mylar membrane, thus shortening the positive phase duration of the compressed air-driven mode of the MBD without significantly altering the peak overpressure magnitude \[128\]. Additionally, the positive phase duration of any mode of the MBD can be lengthened without significantly altering the peak overpressure magnitude by the use of reflective surfaces positioned outside the open end of the shock tube that reflect the shockwave back into the open end and thus increase the positive phase duration \[202\]. Evaluation of the injury produced by shockwaves of different pressure-time signatures but from the same source will provide conclusive evidence of the influence of positive phase duration on the injury to the brain by blast.

We have shown pathological differences in the injury produced by shockwaves, however it remains to be seen if shockwaves of different positive phase durations, but equal peak overpressure magnitudes can produce behavioral differences in animals. Behavioral tests that test short- and long-term memory, motor strength and stability, and fear responses should be utilized to determine the effects of positive phase duration on behavioral deficits seen in other modalities of brain injury.
6.4 Concluding Remarks

The mechanisms by which blast injures the brain are still incompletely understood. Though some advances in understanding the pathology from blast waves have been made, the contribution of various shockwave components to the injury has remained largely unstudied. Because of the documented differences between shockwaves of different sources, it is important to be aware of the potential for differential injury due to specific situations which can give rise to blast waves of different pressure-time signatures.

The results of the studies outlined in this dissertation have far-reaching implications for the diagnosis and treatment of blast-induced brain injury. Previous reports of the shockwaves recorded during the injury of animal test subjects by compressed air- or chemical explosive-driven shockwaves have shown the injury due to different shockwave pressure-time signatures [124, 126, 203], however no studies have examined the shockwaves produced form different sources within the same device. These results would have been difficult to obtain from other blast injury devices due to the fact that they used a single driving source. Prior to the development of studies outlined in Chapter 2, it would have been much more difficult to determine the contribution of various shockwave components to brain injury.

Vascular damage associated with blast-induced brain injury and long-term cognitive sequelae of blast injury that could be associated with mechanisms outlined in Chapter 4 have been reported in soldiers exposed to blast [68, 77, 153], while most blast injury research reports focus on positive magnitude as a determining factor for injury [24,
The results of Chapters 3 and 4 of this dissertation suggest a substantial role for the positive phase in both immediate and long-term consequences of blast-induced brain injury. Our results indicate that a change in the way blast-induced brain injury researchers should take into account positive phase duration when drawing conclusions about the injuries sustained by animals subjected to shockwaves of different sources should be considered. Additionally, as we have reported a contribution of an additional component of the shockwave to injury, there are other components of the shockwave (i.e., heat, acoustic, electromagnetic) which should be evaluated for their contribution to the injury.

Current military armor systems are designed to protect against ballistics, projectiles, and conventional mechanisms of TBI, however their blast wave mitigation ability has not been evaluated [204, 205]. Body armor has been suspected to intensify the blast injury in two ways [206]. First, the body armor may act as an improved surface for shock front/body interaction, thus increasing energy transfer [68]. Secondly, armor may also serve as a surface for reflection and concentration of the blast wave, reflecting the blast wave as it resonates internally [207]. Computer simulations aimed at spatiotemporal prediction of blast wave propagation inside the helmet and through the skull predict that helmets may serve to amplify the blast wave pressure on the skull and brain [170, 208, 209]. Whether or not blast waves are amplified by the helmet remains to be verified; however reflected blast waves can have significantly increased positive phase durations and elevated peak pressures compared to the primary wave from which they came due to the phenomenon of rarefaction [96]. In addition to amplification on the skull by the helmet, reflected blast waves can be encountered by soldiers in enclosed spaces.
following blasts occurring either inside or outside the structure or vehicle in which the soldiers are positioned. It has long been assumed that the positive phase duration of the shockwave is important to the brain injury it produces [139]. However, there has been no experimental examination of the positive phase’s contribution to the injury to date. It may be possible to design armor that protects from the peak magnitude while not increasing the positive phase duration through the use of vents that allow trapped pressure to be released instead of accumulating. Alternatively, structures in high-risk areas or armor could be designed to slow the blast wave propagation to non- or less-injurious velocities.

The differences in injury caused due to an increased positive phase outlined in this dissertation will be of importance to physicians when evaluating a blast victim for injury severity. Additionally, with the wealth of mechanistic and drug design study information constantly coming to light, treatments for individual facets of both the immediate and long-term secondary injury cascade are most certainly imminent. This leads to the possibility of patient-tailored drug and treatment regimens that focus on treating the type of blast wave to which a person was exposed. Engineers and designers of shockwave mitigation technologies may also need to consider other components of the blast wave when designing armor, vehicles, and structures at risk for blast exposure. Overall, the data presented in this dissertation aid in the understanding of mechanisms of blast-induced brain injury and in future prevention and treatment.

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Appendix A: List of acronyms and abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BBB</td>
<td>Blood-brain Barrier</td>
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<td>BCA</td>
<td>Bicinchoninic Acid</td>
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<td>BS</td>
<td>Brain Stem</td>
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<tr>
<td>bTBI</td>
<td>Blast-induced Traumatic Brain Injury</td>
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<tr>
<td>C-4</td>
<td>Composition C-4 (plastic explosive)</td>
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<tr>
<td>CBM</td>
<td>Cerebellum</td>
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<tr>
<td>CCI</td>
<td>Controlled Cortical Impact</td>
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<td>CDX</td>
<td>Contralateral Dorsal Cortex</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>CO</td>
<td>Carbon Monoxide</td>
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<td>CPX</td>
<td>Contralateral Piriform Cortex</td>
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<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<td>CSPG</td>
<td>Condroitin Sulfate Proteoglycan</td>
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<td>CVPL</td>
<td>Contralateral Ventral Posteriolateral Nucleus</td>
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<tr>
<td>CVX</td>
<td>Contralateral Ventral Cortex</td>
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<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
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<tr>
<td>Δ Impulse</td>
<td>Impulse Difference</td>
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<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
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<tr>
<td>DOT</td>
<td>Department of Transportation</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbtant Assay</td>
</tr>
<tr>
<td>ft</td>
<td>Foot (unit of length equal to 0.3048 meters)</td>
</tr>
<tr>
<td>g</td>
<td>Gram (metric unit of mass)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>H2-O2</td>
<td>Oxyhydrogen (2:1 mixture, respectively, of hydrogen and oxygen)</td>
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<tr>
<td>H2+O2</td>
<td>Oxyhydrogen (2:1 mixture, respectively, of hydrogen and oxygen)</td>
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<td>Iba1</td>
<td>Ionized Calcium Binding Adapter Molecule 1</td>
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<td>ICP</td>
<td>Intracranial Pressure</td>
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<td>IED</td>
<td>Improvised Explosive Device</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<td>Ipsilateral Dorsal Cortex</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IVPL</td>
<td>Ipsilateral Ventral Posteriolateral Nucleus</td>
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<tr>
<td>IVX</td>
<td>Ipsilateral Ventral Cortex</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram (One thousand grams)</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilopascal</td>
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<tr>
<td>MBD</td>
<td>McMillan Blast Device</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram (One thousandth of a gram)</td>
</tr>
<tr>
<td>mil</td>
<td>Thousandths of an Inch</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
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O₂ – Oxygen
OEF/OIF – Operations Enduring Freedom and Iraqi Freedom
rcf – Relative Centrifugal Force (equal to 1 X gravitational force)
RDX – Research Department Explosive (cyclotrimethylenetrinitramine)
RIPA – Radio-immunoprecipitation Assay
SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sec – Seconds
TBI – Traumatic Brain Injury
TBS – Tris-Buffered Saline
TTBS – Tris-Buffered Saline Containing 0.05% Tween-20
TNF-α – Tumor Necrosis Factor Alpha
VPL – Ventral Posteriolateral Nucleus
w/v – Weight Per Volume (Defined as 100g/100mL Solution)
Appendix B. Western blot analysis of blood-brain barrier compromise following shockwave exposure

A. IVX IgG Content

B. CBM IgG Content

C. BS IgG Content

Appendix B. Western blot analysis of blood-brain barrier compromise between shockwave sources. A. IgG content of IVX tissue taken from sham-, oxyhydrogen-, and compressed air-exposed rats. B. IgG content of CBM tissue taken from sham-, oxyhydrogen-, and compressed air-exposed rats. C. IgG content of BS tissue taken from sham-, oxyhydrogen-, and compressed air-exposed rats. There was no significant increase in IgG content following shockwave exposure from either source detectable in IVX, CBM, or BS samples by Western blot following shockwave exposure from either source. One-way ANOVA, n=4 per treatment group.
Appendix C: Correlation between IgG extravasation and peak blast overpressure or impulse in the cerebellum

Appendix C. CBM IgG:Actin ratios as a function of peak overpressure and impulse exposure.  A. Peak blast overpressure exposure vs. IgG:Actin ratio separated by shockwave source. There was no positive effect of blast overpressure on IgG extravasation for animals exposed to compressed air- or oxyhydrogen-driven shockwaves.  B. Peak blast impulse exposure vs. IgG:Actin ratio separated by shockwave source. There was no positive effect of impulse on IgG extravasation for animals exposed to compressed air- or oxyhydrogen-driven shockwaves.  C. Peak blast overpressure exposure vs. IgG:Actin ratio. There was no relationship between pressure and IgG extravasation.  D. Peak blast impulse exposure vs. IgG:Actin ratio. There was no relationship between impulse and IgG extravasation. Pearson correlation, two-tailed. n=6 or 19 (A & B) or n= 25 (C & D) per treatment group.
Appendix D: Correlation between IgG extravasation and peak blast overpressure or impulse in the brain stem

Appendix D. BS IgG:Actin ratios as a function of peak overpressure and impulse exposure.  A. Peak blast overpressure exposure vs. IgG:Actin ratio separated by shockwave source.  There was no positive effect of blast overpressure on IgG extravasation for animals exposed to compressed air- or oxyhydrogen-driven shockwaves.  B. Peak blast impulse exposure vs. IgG:Actin ratio separated by shockwave source.  There was no positive effect of impulse on IgG extravasation for animals exposed to compressed air- or oxyhydrogen-driven shockwaves.  C. Peak blast overpressure exposure vs. IgG:Actin ratio.  There was no relationship between pressure and IgG extravasation.  D. Peak blast impulse exposure vs. IgG:Actin ratio.  There was no relationship between impulse and IgG extravasation.  Pearson correlation, two-tailed. n=6 or 19 (A & B) or n= 25 (C & D) per treatment group.
Appendix E. Western blot analysis of astrocytosis following shockwave exposure.

A. GFAP content of IVX tissue taken from sham-, oxyhydrogen-, and compressed air-exposed rat IVX (A), CVX (B), IDX (C), CDX (D), CBM (E), or BS (F). There was no significant increase in IgG content following shockwave exposure from either source detectable in IVX, CVX, IDX, CDX, CBM, or BS samples by Western blot following shockwave exposure from either source. One-way ANOVA, n=4 per treatment group.
Appendix F: Western blot analysis of microgliosis following shockwave exposure

Appendix F. Western blot analysis of microgliosis between shockwave sources. GFAP content of tissue taken from sham-, oxyhydrogen-, and compressed air-exposed rat IVX (A), IDX (B), CBM (C), or BS (D). There was no significant increase in IgG content following shockwave exposure from either source detectable in IVX, IDX, CBM, or BS samples by Western blot following shockwave exposure from either source. One-way ANOVA, n=4 per treatment group.
Appendix G – Anterior-posterior distribution of rod-shaped microglia at 3h post injury.

Rod-shaped microglia were found in the entire anterior-posterior distribution of the piriform cortex on both the ipsilateral (IPX; blast side) and contralateral (CPX; side opposite blast) sides of the brain (A-B). Higher numbers of rod-shaped microglia were found in the middle two sampled piriform cortices of compressed air-injured rat brains compared to sham- and oxyhydrogen-injured rat brains (A-B). Though sporadic rod-shaped microglia were present in the entire dorsolateral thalamus of compressed air-injured animals, they were most concentrated in the ventral posteriolateral nuclei of the ipsilateral and contralateral (IVPL and CVPL, respectively) sides of the brains of rats exposed to compressed air-driven shockwaves.
Appendix H – Anterior-posterior distribution of rod-shaped microglia at 24h post injury

Appendix G – Anterior-posterior distribution of rod shaped microglia at 24h post injury. Rod-shaped microglia were found in the entire anterior-posterior distribution of the piriform cortex on both the ipsilateral (IPX; blast side) and contralateral (CPX; side opposite blast) sides of the brain (A-B). Higher numbers of rod-shaped microglia were found in the middle two sampled piriform cortices of compressed air-injured rat brains compared to sham- and oxyhydrogen-injured rat brains (A-B). Though sporadic rod-shaped microglia were present in the entire dorsolateral thalamus of compressed air-injured animals, they were most concentrated in the ventral posteriolateral nuclei of the ipsilateral and contralateral (IVPL and CVPL, respectively) sides of the brains of rats exposed to compressed air-driven shockwaves.
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University of Kentucky College of Medicine, Department of Microbiology, Immunology and Molecular Genetics.

Research Intern
University of Kentucky College of Arts and Sciences, Department of Biology.
Dates: January 2005.

Research Assistant
Centre College, Department of Biology.

Clinical Volunteer
Family Practice of Darby Cole, M.D., Hartford, KY.

PUBLICATIONS

PUBLICATIONS CONT’D


AWARDS/HONORS

• 2012 – Awarded top poster award Cardinal Hill Rehabilitation Hospital Research Day

• 2011 – Awarded 2.5-year individual pre-doctoral training fellowship from the National Institute of Neurological Disorders and Stroke 1F31NS074678.

• 2010 – University of Kentucky Graduate School Award for travel to National Neurotrauma Symposium in Las Vegas, Nevada.

• 2010 – Awarded two-year pre-doctoral fellowship on NIH Training Grant 1T32DA022738.

• 2009 – Awarded two-year pre-doctoral fellowship on Kentucky Spinal Cord and Head Injury Research Trust training grant to the Spinal Cord and Brain Injury Research Center.

• 2006 - American Society for Ricketsiology: Travel Award.

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AWARDS/HONORS CONT’D

• 2001 - Boy Scouts of America: Eagle Scout.