REVIEW

The pig as a preclinical traumatic brain injury model: current models, functional outcome measures, and translational detection strategies

Holly A. Kinder[1, 2], **Emily W. Baker**[1, 2], **Franklin D. West**[1, 2, *]
1 Regenerative Bioscience Center, University of Georgia, Athens, GA, USA
2 Department of Animal and Dairy Science, University of Georgia, Athens, GA, USA

Abstract

Traumatic brain injury (TBI) is a major contributor of long-term disability and a leading cause of death worldwide. A series of secondary injury cascades can contribute to cell death, tissue loss, and ultimately to the development of functional impairments. However, there are currently no effective therapeutic interventions that improve brain outcomes following TBI. As a result, a number of experimental TBI models have been developed to recapitulate TBI injury mechanisms and to test the efficacy of potential therapeutics. The pig model has recently come to the forefront as the pig brain is closer in size, structure, and composition to the human brain compared to traditional rodent models, making it an ideal large animal model to study TBI pathophysiology and functional outcomes. This review will focus on the shared characteristics between humans and pigs that make them ideal for modeling TBI and will review the three most common pig TBI models–the diffuse axonal injury, the controlled cortical impact, and the fluid percussion models. It will also review current advances in functional outcome assessment measures and other non-invasive, translational TBI detection and measurement tools like biomarker analysis and magnetic resonance imaging. The use of pigs as TBI models and the continued development and improvement of translational assessment modalities have made significant contributions to unraveling the complex cascade of TBI sequela and provide an important means to study potential clinically relevant therapeutic interventions.

Key Words: *traumatic brain injury; large animal model; pig model; diffuse axonal injury; functional outcome assessment measures; controlled cortical impact model; fluid percussion injury model; magnetic resonance imaging, biomarkers*

***Correspondence to:**
Franklin D. West, PhD,
westf@uga.edu.

orcid:
0000-0002-0504-7997
(Franklin D. West)

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Introduction

Traumatic brain injury (TBI) is a leading cause of long-term disability and death. It has been estimated that as many as 1.7 million people sustain a TBI annually in the United States, and almost one third of all head injuries occur among children aged 0 to 14 (Langlois, 2006). TBI can be caused by a multitude of factors such as falls, blast waves, motor vehicle accidents, rapid acceleration and deceleration, or penetration of a foreign object (Maas et al., 2008). Brain injuries are generally considered focal or diffuse in nature, but may oftentimes involve elements of both, such as a focal contusion with associated diffuse axonal injury (Andriessen et al., 2010). TBI can lead to immediate and sustained impairments in physiologic and hemodynamic responses that perpetuate secondary injury progression. The secondary injury cascade is characterized by the release of pro-inflammatory cytokines, chemokines, free radicals, mitochondria dysfunction, and oxidative stress that contribute to cell death, axonal degeneration, tissue necrosis, and ultimately, functional impairments (Kenney et al., 2016; Ichkova et al., 2017). Animal models of TBI have proven to be important in characterizing the pathophysiology of injury progression and in developing effective management strategies (Xiong et al., 2013). The pig model has recently come to the forefront due to similarities in brain size, structure, composition, and development to humans (Dobbing and Sands, 1979; Flynn, 1984; Gieling et al., 2011; Conrad et al., 2012). A number of diverse experimental models of TBI have been developed in pigs to recapitulate and study different aspects of human brain injury including the diffuse axonal injury (DAI), the controlled cortical impact (CCI), and the fluid percussion injury (FPI) models (Duhaime, 2006). Studies using these pig TBI models have provided important evidence detailing TBI pathophysiology and potential therapeutic targets.

TBI can lead to significant functional impairments that affect cognition, motor function, and overall quality of life (Koskinen, 1998; Anderson et al., 1999, 2012; Williams et al., 2009). Cognitive deficits generally correlate with injury severity. Mild TBI is typically associated with few, if any, cognitive deficits which normally resolve within three months in human patients (Mathias and Coats, 1999; Belanger et al., 2005). Although, there is some evidence of a small cohort of patients that experience persistent cognitive impairments even after mild TBI (Roe et al., 2009). Moderate to severe TBI is associated with more complex and long-term cognitive deficits that can remain unresolved for months or up to the lifetime of the patient (Rabinowitz and Levin, 2014). Similarly, motor function deficits have been found to correlate with injury severity and can impair both fine and gross motor function abilities (Kuhtz-Buschbeck et al., 2003). Increased interest in studying functional changes after TBI in animal models has led to a small number of pig TBI studies that assess functional outcomes (Friess et al., 2007; Sullivan et al., 2013b; Baker et al., 2018). Although the

field is lacking in well-validated behavioral measures to assess neurocognitive and motor function changes in pig TBI models, these initial studies show the importance of identifying and characterizing functional outcome measures after experimental TBI in large animal models.

In this review, we will discuss the brain anatomy and physiology and functional characteristics that make the pig ideal for use as a large animal model. We will discuss the DAI, CCI, and FPI pig models and review the limited, albeit important, initial studies that performed neurobehavioral and motor function testing in pig TBI models to assess cognitive and motor function changes. Finally, we will discuss the progress made in utilizing translational, non-invasive biomarker and magnetic resonance imaging (MRI) detection strategies to longitudinally measure TBI progression and recovery mechanisms.

The articles in this review were retrieved using the following electronic databases: PubMed/MEDLINE, Web of Science, and Google Scholar. The search was limited to articles published between January 1, 1950 and September 1, 2018. Search terms were identified in the title, abstract, and key words using (pig OR porcine OR swine) AND each the following search terms: TBI, diffuse axonal injury, controlled cortical impact, fluid percussion injury, behavior, cognition, gait, motor function, neurologic score, biomarkers, magnetic resonance imaging.

Advantages of Pigs in Modeling TBI

Anatomical and physiologic characteristics of pigs

The pig possesses a number of advantageous characteristics that make it suitable for modeling brain disorders like TBI. The size, organization, composition, and development of the pig brain is similar to that of the human brain. Compared to adult human brains that weighs between 1300–1400 g, adult pig brains, depending on breed, weigh between 80–180 g (Gieling et al., 2011). This is in vast contrast to mature mouse or rat brains that weigh approximately 0.5 and 2 g, respectively (Hofman, 1985). Brain size is important in modeling TBI such that injury type, location, or severity leads to appropriate injury responses that are similar to that of human TBI. For example, in rodents, the hippocampus is more vulnerable to focal cortical injury due to smaller brain size combined with the location and more superior orientation of the hippocampus (Holm and West, 1994; Strange et al., 2014). This is in contrast to human and pig brains in which the hippocampus lies more ventrally and deep within the temporal lobe and is thus more protected from injury (Holm and West, 1994; Strange et al., 2014).

A number of studies have investigated changes in myelination in the pig brain during brain maturation, noting a similar time course of myelination when compared to humans (Prensky et al., 1971; Flynn, 1984; Pond et al., 2000). Fang et al. (2005) used MRI and histological analysis to show that myelination in the pig brain increases through adolescence up to 6 months of age when the pig reaches sexual maturity. Similarly, in humans myelination can continue through adolescence and even into early adulthood (Giedd et al., 1999). This period of myelination is critical for normal brain function and is therefore recognized as being a vulnerable period in development (Davison, 1977). Brain injury such as TBI can also contribute to widespread white matter damage in young children that can lead to long-term neurological deficits (Genc et al., 2017). Despite the high prevalence of rodent TBI models, the myelination pattern of rodents is different from that of humans with significant differences in both time scale of myelination and total amount of white matter (Norton and Poduslo, 1973). For example, myelination in the rat brain is mostly complete by 3 months of age, and the mature rat brain is comprised of less than 12% white matter, while the mature human and pig brain are comprised of more than 60% white matter (Zhang and Sejnowski, 2000; Ahmad et al., 2015; Hammelrath et al., 2016). White and gray matter have been found to have different mechanical properties; gray matter has less anisotropy and is stiffer compared to white matter (Prange and Margulies, 2002). Therefore, use of an animal model like the pig with a similar white to gray matter ratio is ideal for modeling TBI.

The organization of the pig brain resembles that of the human brain. Pig brains, like human brains, are gyrencephalic and follow a similar gyral pattern (Gieling et al., 2011). Rodent brains, however, lack gyri and sulci, and are thus lissencephalic. The volume of the prefrontal cortex in Göttingen minipigs constitutes 10% of total brain volume which is comparable to humans whose prefrontal cortex constitutes approximately 12.5% of total brain volume (McBride et al., 1999; Jelsing et al., 2006). Anatomically, the dorsal striatum in pig brains is split by the internal capsule into two distinct structures- the caudate nucleus and putamen; in comparison, rodent brains contain a single caudate-putamen structure (Matsas et al., 1986; Félix et al., 1999). The pig hippocampus has been well described and has been found to be structurally more similar to the human hippocampus than the rodent, having a degree of encephalization that lies between rodent and primate (Holm and West, 1994). A number of descriptive, comparative anatomical studies have been performed for the pig brain thalamus, hypothalamus, hypothalamic nuclei (Campos-Ortega, 1970; Junge, 1977; Szteyn et al., 1980), brainstem structures (Freund, 1969; Otabe and Horowitz, 1970), and cerebellum (Larsell, 1954). Similar to humans, sensory cortices such as the motor cortex (Breazile, 1966) and somatosensory cortex (Craner and Ray, 1991) are arranged somatotopically. Brain anatomy and organization is important in modeling TBI as it effects the brain regions injured, vascular responses, and ultimately, the short- and/or long-term clinical effects of injury (Duhaime, 2006).

Use of an animal model with similar pathophysiologic responses to injury is a key component to the development of efficacious TBI therapeutics. Though pig and human brains are certainly not identical in their response to injury, the pig brain does possess a number of advantageous characteristics that suggest it may have a more human-like pathophysiologic response to injury compared to small animal models. The pig immune system and inflammatory responses may be more similar to humans after injury (Fairbairn et al., 2011;

Dawson et al., 2013, 2017). Toll like receptors (TLRs) are present on various innate immune cells such as microglia within the brain and have been implicated to play a significant role in inflammatory responses after injury (Crack and Bray, 2007). Pigs and humans both have ten different TLRs (TLR1–10) compared to twelve in mice (TLR1–9, 11–13), have more similar TLR promoter sequences (71% similarity between pigs and humans compared to 53% similarity between mouse and humans for TLR4), and share typical functional domains (Thomas et al., 2006; Kawai and Akira, 2010; Mair et al., 2014). An immunological comparison between humans, pigs, and mice revealed that, compared to mice, the pig immune system is more similar to humans for over 80% of immune system variables compared (Schook et al., 2005). In addition, the pig immune system appears to have a more human-like inflammatory response to immune challenge (Dawson et al., 2017). For example, human and pig microglial cells fail to generate the toxic radical nitric oxide after cytokine stimulation in response to lipopolysaccharide *in vitro*, unlike murine microglial cells (Hu et al., 1996).

With respect to vasculature, pig and human cerebrovascular anatomy, vascular outflow systems (Habib et al., 2013; Mancini et al., 2015), and cerebral blood flow rates (Harada et al., 1991; Xu et al., 2011), though not identical, are more comparable than rodents. This is likely due to more comparable brain size and gyrencephalic structure. As such, pig and human brains exhibit shared characteristics in terms of vascular responses to cerebral insult and subsequent revascularization processes (Nakamura et al., 2009). Microvasculature in gray and white matter components are unique. Grey matter is 3 to 5 times more vascularized than white matter and this lack of collateralization and redundancy leads white matter to be more susceptible to injury (Nonaka et al., 2003; Matute, 2010). Similar to humans, pigs have been shown to be especially susceptible to white matter injury given their high proportion of white to gray matter (McGowan et al., 1999). Direct comparisons of pig and human brain tissue pathophysiologic responses to injury are lacking, therefore future studies that more clearly delineate between the similarities and differences in injury responses between species will be integral to the development of effective therapies.

Pig Traumatic Brain Injury Models

The DAI, CCI, FPI models are the most common pig models developed to study temporal pathophysiological mechanisms and functional outcomes with the goal of translating preclinical findings to a clinical setting (**Additional Table 1**). Experimental TBI models have also provided an important means to develop and refine TBI detection and measurement modalities that can be used to provide critical insight in identifying therapeutic targets and to test the efficacy of potential treatments (**Figure 1**).

Diffuse axonal injury model

DAI in humans occurs as result of damage to axons in brain neural tracts, specifically in major white matter tracts and can lead to significant morbidity and even mortality (Meythaler et al., 2001). DAI is caused by acceleration and deceleration inertial forces that lead to disruption of neurofilament subunits within the axonal cytoskeleton (Meythaler et al., 2001). This type of injury is multifocal and predominantly restricted to axons of white matter tracts (Wang and Ma, 2010). DAI in pigs has typically been generated by a rapid nonimpact rotational injury by securing an anesthetized animal's head in a rotational acceleration injury apparatus that can produce either single (Raghupathi and Margulies, 2002) or repetitive (Raghupathi et al., 2004; Friess et al., 2009) impulsive rotation in multiple (*e.g.*, coronal) planes. Injury severity can be controlled by manipulating the acceleration

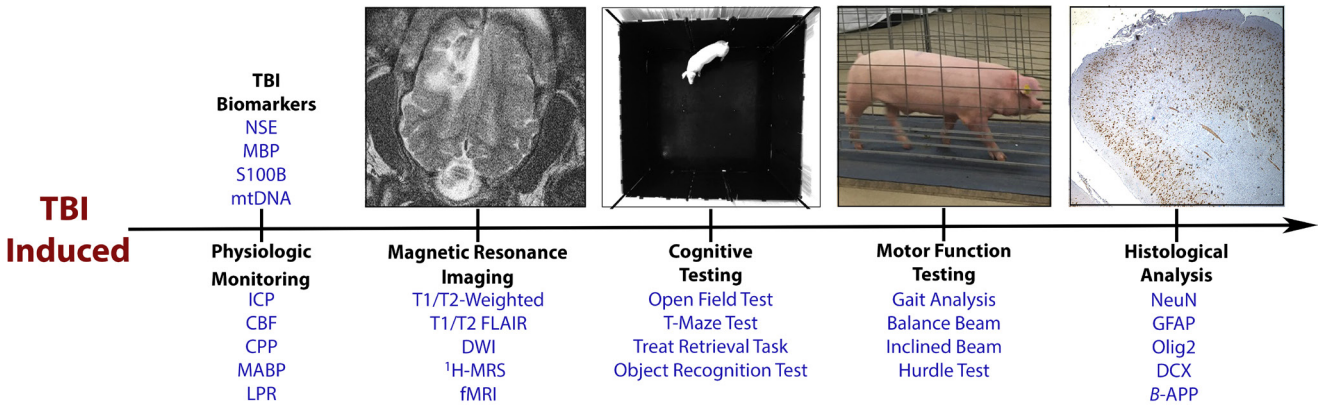

Figure 1 Major assessment modalities utilized to evaluate pathophysiology and functional outcomes in pig traumatic brain injury (TBI) models.

Serum biomarkers, physiologic assessments, magnetic resonance imaging, cognitive testing, motor function testing, and histological analyses provide a platform with which to test TBI related hypotheses. The combination of these assessment modalities enables comprehensive evaluation of unique molecular and cellular TBI pathophysiology and the manifested tissue and functional changes. These tools are also invaluable in identifying potential treatment targets and testing the efficacy novel therapeutics. NSE: Neuron-specific enolase; MBP: myelin basic protein; mtDNA: mitochondrial DNA; LOC: loss of consciousness; ICP: intracranial pressure; CBF: cerebral blood flow; CPP: cerebral perfusion pressure; MABP: mean arterial blood pressure; LPR: lactate pyruvate ratio; T1/T2 FLAIR: fluid-attenuated inversion recovery; DWI: diffusion-weighted imaging; ^{1}H-MRS: magnetic resonance spectroscopy; fMRI: functional magnetic resonance imaging; NeuN: neuronal nuclei; GFAP: glial fibrillary acidic protein; DCX: doublecortin; β-APP: β-amyloid precursor protein.

velocity and directionality to generate injuries ranging from mild to severe (Ibrahim et al., 2010; Browne et al., 2011).

DAI in humans can often lead to temporary or sustained loss of consciousness and apnea and can influence systemic physiologic responses (Meythaler et al., 2001; Vieira et al., 2016). Similarly, DAI in pigs can result in immediate loss of consciousness (Raghupathi and Margulies, 2002; Friess et al., 2011). Presence and length of loss of consciousness is dependent on direction of rotational acceleration and number of rotations. Browne et al. (2011) demonstrated that rotational injury in the axial but not coronal plane results in loss of consciousness. Eucker et al. (2011) similarly observed no loss of consciousness after rotational injury in the coronal plane, but also showed that rotational injury in the sagittal and horizontal planes did result in loss of consciousness. Friess et al. (2009) showed that repetitive rotational injury led to a significant increase in duration of unconscious time compared to sham animals when rotational injury is 24 hours apart, but not 7 days apart. The incidence of apnea, characterized as cessation of breathing or reduced respiratory effort resulting in arterial oxygen saturation < 90%, was significantly increased after axial (Raghupathi and Margulies, 2002), sagittal (Eucker et al., 2011), and horizontal (Eucker et al., 2011) rotational injury in piglets and is dependent on injury severity (Ibrahim et al., 2010). Rotational injury in pigs may also lead to physiological changes similar to humans including elevated intracranial pressure, decreased cerebral blood flow, decreased cerebral perfusion pressure, decreased brain tissue oxygenation, and elevated lactate-pyruvate ratio (Eucker et al., 2011; Friess et al., 2011). However, mean heart rate, body temperature, respiratory rate, and arterial blood gas appears to remain largely unaffected (Raghupathi and Margulies, 2002; Ibrahim et al., 2010; Browne et al., 2011). Rotational injury can lead to amplitude suppression in EEG recordings (Ibrahim et al., 2010) as well as changes in involuntary auditory event-related potential functional networks (Atlan et al., 2018).

The physiologic responses of DAI in pigs also have been found to have correlative cellular neuropathology comparable to human patients (Wang and Ma, 2010; Friess et al., 2011). Morphologically, traumatically damaged axons in pigs exhibit terminal clubbing (retraction balls) and swelling (Smith et al., 1997; McGowan et al., 1999). The inertial forces of rotational injury disrupt cytoplasm flow leading to intraaxonal damage to neurofilament subunits that impair the axonal cytoskeleton (Meythaler et al., 2001). Neurofilament proteins then disassemble, dephosphorylate, and accumulate in damaged axons (Chen et al., 1999). Accumulation of large neurofilament proteins in the pig brain therefore provides convincing evidence of axonal damage within white matter tracts as a result of injury (Raghupathi and Margulies, 2002; Ibrahim et al., 2010). Accumulation of β-amyloid precursor protein in damaged axons is another hallmark of diffuse injury and has been found to be correlated with injury severity (Ibrahim et al., 2010; Johnson et al., 2013). In addition, accumulation of amyloid-beta and tau proteins have also been observed after rotational injury suggesting a link between TBI and the initiation of the neurodegenerative process (Smith et al., 1999; Chen et al., 2004). The extent of traumatic axonal damage has also been found to correlate with age, such that younger piglets demonstrate a higher density of axon injury and increased intracranial hemorrhage susceptibility in the cerebrum compared to adults after comparable rotational injury (Raghupathi and Margulies, 2002; Weeks et al., 2014). The direction of head motion appears to also play a role in the cellular pathology of DAI. Browne et al. (2011) showed that axial plane injury, but not coronal plane injury, resulted in degenerative neurons in the cortex and hippocampus and greater accumulation of neurofilament protein in damaged axons. Eucker et al. (2011) similarly showed that horizontal and sagittal head rotation but not coronal rotation resulted in significantly higher β-amyloid precursor protein accumulation. These studies demonstrate the potential to focus on key elements of DAI injury such as directionality and repetition and their effects on important relevant physiologic and cellular outcomes in a pig model.

Controlled cortical impact model

As opposed to inertial acceleration-deceleration forces that produce diffuse brain injury, impact forces produce a focal injury that can potentially lead to skull fracture, disruption and hemorrhage of the cortical surface, and contusions (Duhaime, 2006). Impact forces occur when the head comes in contact with a hard surface or object. Falls, for example, account for almost 35% of all sustained head injuries and over 50% of sustained head injuries in children aged 0–14 years (Langlois, 2006). Head injuries incurred from a fall may certainly involve elements of both diffuse and focal TBI, and as a result may be studied in conjunction (Zhang et al., 2008), but it is of interest to experimentally isolate and study the pathophysiological and functional elements of focal injury. As such, the CCI model is a widely utilized and well-validated experimental focal injury model (Osier and Dixon, 2016). A CCI injury is typically induced in an anesthetized animal using a device consisting of an impactor tip that is attached to a shaft that is accelerated by either a pneumatic piston or electromechanical actuator (Osier and Dixon, 2016). Precise, quantitative control over CCI parameters such as velocity, dwell time, and depth of depression allow for scaled manipulation of TBI severity (Osier et al., 2015). In addition, injury location can be targeted to specific brain regions such as the frontal (Manley et al., 2006; Hawryluk et al., 2016) or parietal (Duhaime et al., 2000; Alessandri et al., 2003; Meissner et al., 2011) cortices to generate models with unique deficits. As a result, CCI can produce graded cellular, tissue, physiologic, and functional TBI responses.

Mild to severe TBI can lead to immediate disruption of physiologic responses and impaired autoregulatory function, generally as a function of injury severity (Toth et al., 2016). Similarly, physiologic responses after CCI in pigs have been shown to be sensitive to injury severity, ranging from no physiologic alterations (Duhaime et al., 2000) to significant impairments (*e.g.*, increases in intracranial pressure) (Alessandri et al., 2003; Meissner et al., 2011). Interest-

ingly, Durham et al. (2000) observed an age dependent effect of CCI on cerebral blood flow. They demonstrated that a significant decrease in cerebral blood flow was observed in 1-month- and 4-month-old pigs, but a significant increase in cerebral blood flow in 5-day-old pigs. The authors suggested that this may provide some protection from early ischemia after injury. However, no other physiologic changes were noted. A moderate CCI in adult pigs has been shown to lead to significant physiologic impairments that result in decreases in cerebral perfusion pressure and brain tissue oxygen coupled with significant increases in intracranial pressure, glutamate, and lactate (Alessandri et al., 2003; Meissner et al., 2011). Manley et al. (2006) qualitatively assessed physiologic changes after graded CCI, finding substantial increases in intracranial pressure and heart rate and trending decreases in mean arterial pressure and cerebral perfusion pressure that correlated with increased injury severity. The range in physiologic responses observed after CCI among groups may vary due to a number of key factors including injury severity, age, CCI machinery, pig strain, impactor tip diameter, and injury location. The variation observed between DAI and FPI pig studies are also likely due to similar differences between studies.

In humans, lesion size is frequently utilized as a proxy or biomarker for TBI severity. Duhaime et al. (2000) showed that the lesion volumes of toddler (1-month-old) and adolescent (4-month-old) pigs were significantly larger than infant (5-day-old) pigs in response to proportionally identical injury, suggesting that vulnerability to mechanical trauma increases progressively during maturation. Baker et al. (2018) demonstrated that graded CCI in 4-week-old piglets resulted in increases in brain lesion size that corresponded with increases in depth of depression. In addition to age, gender may also play a contributing role in lesion size for young pigs. TBI male pigs that were 1-month-old developed significantly larger lesions than male 5-day-old pigs, but this effect was not seen in females (Missios et al., 2009).

CCI results in a number of clinically relevant histopathological outcomes including cellular damage/death, inflammation, hemorrhage, swelling and edema (Alessandri et al., 2003; Manley et al., 2006; Baker et al., 2018). Semi-quantitative means to assess histological changes after CCI include the use of graded injury scales that can score relative cell injury, intracerebral bleeding, subarachnoid hemorrhage, edema, and surface contusion (Alessandri et al., 2003; Meissner et al., 2011). Quantitative approaches to assess histological changes after CCI in pigs is limited, but two recent studies by the Duhaime group quantitatively analyzed the proliferation of doublecortin positive neuroblasts at the subventricular zone, their ability to migrate to white and gray matter specific regions, and ultimately target the lesion site (Costine et al., 2015; Taylor et al., 2016). CCI has also been shown to lead to significant increases in ionized calcium binding adapter molecule 1 positive activated microglia at the lesion site (Irvine et al., 2017). Baker et al. quantitatively measured changes in neurons, astrocytes, and oligodendrocytes and found that TBI resulted in a significant loss of neuronal nuclei positive neurons as well as an increase in glial fibrillary acidic protein positive astrocytes that corresponded to injury severity (Baker et al., 2018). Further histological studies are needed in pig CCI models that more quantitatively assess secondary injury mechanisms that contribute to cell death, astrogliosis, microglia activation, and white matter disruption with enhanced spatial and temporal acuity.

Fluid percussion injury model

FPI has elements of both diffuse and focal injury. There are two main types of FPI-medial and lateral. A medial injury is induced by applying force at the midline, along the sagittal sutures between bregma and lambda, producing a widespread diffuse injury (Kabadi et al., 2010). A lateral injury is induced by applying force over the cerebral cortex, producing a more focal injury targeted to one side of the brain. To generate the injury, a craniotomy is performed to expose the underlying intact dura in an anesthetized animal. Then using a fluid percussion device, a pendulum strikes the piston of a reservoir of fluid, generating a fluid pressure pulse that impacts the extradural space producing a brief displacement of brain tissue (Xiong et al., 2013). Injury severity corresponds to the strength of the pressure pulse and results in graded pathophysiological and functional changes (Sullivan et al., 1976).

In the immature pig, it is well established that FPI contributes to age-dependent hemodynamic effects (Armstead, 2000). Armstead showed that moderate FPI decreased systemic arterial blood pressure in newborn piglets, but increased it in juvenile piglets (Armstead and Kurth, 1994). Pial arteries constricted to a greater extent, regional cerebral blood flow remained restricted for a longer period of time, and cerebral oxygenation, an index of metabolism, was transiently increased and followed by a prolonged decrease in newborn piglets relative to juvenile piglets (Armstead and Kurth, 1994). In addition, upregulation of endothelin-1 activity after FPI has been found to contribute to adenosine triphosphate-sensitive potassium (K^+) channel impairments that are age-dependent, leading to impaired hypotensive cerebral autoregulation (Armstead, 1999a, b; Armstead and Kreipke, 2011; Armstead and Raghupathi, 2011). Further, disruption of cerebral hemodynamics after injury may be due to impairments in N-methyl-D-aspartate receptor mediated vascular dilation, which has also been found to be age-dependent (Armstead, 2004). Recent evidence has shown that the dopaminergic system may be sensitive to FPI. Acute increases in dopamine can have potentially neurotoxic effects and has been shown to play a role in the pathogenesis of neuronal injury, potentially through the production of free radicals (Walter et al., 2004). Measurement of aromatic amino acid decarboxylase levels, an indicator of dopamine activity, has been shown to be upregulated in newborn piglets but not juvenile piglets after FPI (Walter et al., 2004). Disruption of cholinergic neurotransmission, evident by increased acetylcholinesterase activity (Donat et al., 2010b) and reduced muscarinic Ach receptor density (Donat et al., 2010a), has been observed after FPI. This has

led to depressed cholinergic transmission that can ultimately contribute to the development of functional impairments.

Contusive TBI in humans activates secondary injury mechanisms including inflammation, apoptosis, and necrotic cell death (Werner and Engelhard, 2007). Similarly, immunohistological analyses after FPI in pigs have revealed hemorrhage (Kim et al., 2014), neuronal necrosis in the cortex (Armstead and Raghupathi, 2011; Kim et al., 2014) and hippocampus (Armstead et al., 2016; Bohman et al., 2016) as well as dramatic increases in activated microglia associated with amyloid precursor protein positive axonal swellings (Lafrenaye et al., 2015). These studies suggest that FPI in pigs recapitulates both diffuse and focal aspects of TBI in a more similar gyrencephalic brain and leads to severity-dependent changes in physiologic responses, cerebral hemodynamics, and histological changes.

Functional Measures in Pigs

Changes in cognition, behavior, and motor function after TBI

Cognitive and motor function deficits in both children and adults due to TBI have been well chronicled (Rabinowitz and Levin, 2014). TBI location, severity, and age of patient are key parameters in determining whether the effects are short- or long-term as well as the degree of recovery (Novack et al., 2001; Kinnunen et al., 2011). For example, children who sustain a TBI at a younger age are more likely to develop cognitive dysfunction, likely as a result of disruption of the normal developmental processes during a critical time period of brain maturation (Anderson et al., 1999; Babikian and Asarnow, 2009). Therefore, it is important to study injury effects on cognitive and motor function and identify potential key points of intervention. To date, a large number of rodent studies have investigated cognitive and motor function changes after TBI (Hamm et al., 1992; Ajao et al., 2012; Kamper et al., 2013; Peterson et al., 2015; Tucker et al., 2016). However, important similarities between humans and pigs may make them better suited for studying functional brain responses to injury and potential treatments (Gieling et al., 2011).

Only a limited number of studies have performed a comprehensive assessment of neurobehavioral functional changes after TBI in pigs. Friess et al. first utilized several behavioral tests, namely a modified open field test, a glass barrier task, and a food cover task after mild and moderate rotational injury in piglets (Friess et al., 2007). They found moderately injured pigs exhibited limited changes in open field behavior, although injured pigs spent significantly less time sniffing the walls of the arena than sham pigs. In the glass barrier task and food cover tasks, injured pigs had significantly higher failure rates than sham pigs on the first day of testing only. In a follow-up study, Friess et al. (2009) subjected piglets to either single or repetitive (24 hours and 1 week) rotational injury and found that repetitive injury after 24 hours resulted in significantly higher failure rates of the glass barrier task on day 1. Overall, these early cognitive studies showed minor, transient changes after TBI, yet laid the groundwork for future studies.

Noting the presence of high variability and possible lack of sensitivity of these tests, Sullivan et al. (2013a, b) made a series of changes to several behavior tests to improve their reliability and sensitivity. First, they used improved open field measures to assess locomotion and exploratory behaviors after injury to assess the effect of rotational direction (axial *vs*. sagittal) on cognitive function (Sullivan et al., 2013a). Open field measures indicated that pigs with sagittal rotation injury had a significantly greater level of inactivity and less random use of the open field arena than pigs with axial rotation injury (Sullivan et al., 2013a). To improve the sensitivity of the T-maze test, Sullivan et al. (2013b) added visual discrimination cues. Injured pigs that showed a significant increase inactivity in open field testing showed increased latency to correct choice after reversal in T-maze test. The authors suggest this is likely due to deficits in visual cue based learning.

Multiple groups have developed food retrieval tasks to assess memory retention, recall, color/pattern recognition, spatial memory, and prioritization. Oldland et al. (2012) used a treat retrieval test to evaluate the ability of adult pigs to recognize a food-containing bin when presented with three identical bins and Irvine et al. (2017) used a bucket memory test in which pigs were trained to discriminate between three patterned buckets containing a food reward. However, no impairments in memory were observed after CCI using either treat retrieval tasks. The Alam group developed a more advanced food retrieval task with color coded boxes to test recovery of CCI animals with and without treatments (Halaweish et al., 2015; Georgoff et al., 2017; Williams et al., 2018). Pigs were taught to retrieve a food reward from a specific colored box with food reward without opening any of the non-food reward boxes. This novel food retrieval task proved to be sensitive enough to robustly and reliably detect impairments in testing initiation and proficiency in saline-treated CCI animals compared to animals treated with a number of therapeutics including valproic acid, exosomes, lyophilized and fresh plasma (Halaweish et al., 2015; Georgoff et al., 2017; Williams et al., 2018). Treated animals began neurocognitive testing earlier and/or required significantly fewer testing sessions to establish proficiency at the task.

Currently, no "gold-standard" behavior test exists for behavioral and cognitive assessments in pig TBI models due to lack of reliability and validation of existing tests. In rodent TBI models, the current gold-standard behavioral assessment is the Morris water maze test (Washington et al., 2012). However, the Morris water maze test is not a realistic option for cognitive testing in pigs given the challenges associated with making a water maze large enough for pigs. The open field test is also highly utilized to assess behavioral changes after TBI in rodents (Budinich et al., 2013). Continued refinement of the open field test in pig TBI models, such as through the use of automatic tracking software, may be advantageous given that it has been proven to provide accurate, quantifiable measures of behavioral changes in other pig models (Donald et al., 2011; Webb et al., 2018). In addition, further refine-

ment of the traditional T-maze test, which can be confounded by intrinsic, egocentric mechanisms, may be improved by use of a plus T-maze to provide a more accurate measure of spatial memory formation more similar to the Morris water maze test (Elmore et al., 2012). Given the success of treat retrieval tests in detecting memory impairments in TBI pigs, it is of interest for future studies to utilize this and related tests. Detecting small changes in colors and patterns can be challenging for pigs, which may make acquisition of this task more difficult and thus potentially more sensitive to minor, TBI-induced changes (Tanida et al., 1991).

In an effort to develop more robust and sensitive readouts, a number of groups have developed unique scales combining a number of functional readouts. Friess et al. (2009) developed a cognitive composite dysfunction score which consolidated behavioral outcomes. They found that cognitive composite dysfunction scores correlated well with percent white matter injury (r = 0.83) and were significantly higher after repetitive TBI than single TBI. Sullivan et al. (2013b) developed a sensitive composite porcine disability score using a number of different significant metrics (*e.g.*, open field sniffing walls, inclined beam time, T-maze trial time) from a motor proficiency score (MPS) and the open field, inclined beam, and T-maze tests. The porcine disability score test showed small but persistent impairments in cognition that correlated with percent axonal injury. The Alam group developed a neurological severity score system (Halaweish et al., 2015; Georgoff et al., 2017; Williams et al., 2018). The neurological severity score is a 32-point scale (where 0 is no deficit and 32 is severe deficits) that measures level of consciousness, behavior, appetite, standing position, head position, utterance, gait, and motor function. Saline-treated CCI animals were found to have a significantly higher neurological severity score and/or a longer time to complete neurological recovery compared to CCI animals treated with a number of therapeutics including valproic acid, exosomes, lyophilized and fresh plasma. Future studies are needed to compare and validate existing behavior tests, to identify the most sensitive and predictive assessments, and to develop unique tests to assess different aspects of cognition.

Assessments of motor function impairments in pig TBI models are also limited. A balance beam test and a hurdle cross test have been utilized to measure gross changes in gait and balance after TBI, although no deficits in motor function were observed (Friess et al., 2007; Friess et al., 2009; Irvine et al., 2017). To improve the balance beam test, Sullivan et al. (2013b) inclined the beam and developed a motor proficiency score (MPS) that ranged from gait abnormalities on every run (score of 0) to no gait abnormalities on all runs (score of 4). Although the MPS was significantly lower for injured pigs compared to sham pigs 1 day post-TBI, gait deficits were only transient in nature. However, a 0–4 ranking scale of motor function can only provide a gross assessment of improvement and lacks the resolution to give quantitative measures of specific spatiotemporal gait parameters. Recently, Baker et al. (2018) measured quantitative spatiotemporal gait deficits after graded CCI in piglets using high speed cameras and Kinovea gait analysis software. They observed an increase in percent stance time, a decrease in stride velocity, and an increase in 3-limb support time that was correlated to injury severity. They also found that gait impairments were sustained through 7 days post-TBI in more moderately to severely affected pigs (Baker et al., 2018). However, additional studies are needed that provide more comprehensive and quantitative measures of changes in gait and balance after TBI in pig models.

Development of Translational TBI Detection Tools in Pig Models

Pig biomarkers for TBI

Evaluation of serum biomarkers as a means to more easily and rapidly assess TBI severity, predict prognosis, and test the effectiveness of therapeutic intervention has gained interest in recent years in clinical settings, prompting the search for enhanced, more sensitive biomarkers (Kawata et al., 2016). The effectiveness of the peripheral blood biomarkers neuron-specific enolase (NSE), myelin basic protein (MBP), and S100B were tested in pigs aged 5–7 days, 1 month, and 4 months at 15 mintures, 1, 4, and 7 days after a mild CCI injury (Costine et al., 2012). Only NSE was significantly elevated 1 day after CCI in all age groups, although the predictive power of NSE alone was noted to be only poor to fair. Elevated concentrations of NSE have been observed in children and adults after TBI, typically within 8 to 40 hours after injury, and have been found to correlate with injury severity (Berger et al., 2005; Guzel et al., 2008; Park et al., 2018). However, results regarding the ability of NSE to act as a predictor of outcome are mixed, likely due to the effect of injury severity on predictive power (Bandyopadhyay et al., 2005; Thelin et al., 2016). Contrary to the results reported in this study, significantly elevated levels of S100B and MBP have been observed after TBI in children and adults (Berger et al., 2005; Korfias et al., 2007), and S100B has been found to be an important predictor of prognosis (Thelin et al., 2016). However, the lack of elevated S100B or MBP levels after CCI in pigs in the Costine et al. (2012) study may be attributed to the severity of injury induced, such that a mild brain injury may not elicit an elevation in S100B or MBP levels (Geyer et al., 2009). Additional studies in pigs are needed that longitudinally assess serum biomarker responses after graded TBI in order to more closely compare pig biomarker responses to that of humans.

Kilbaugh et al. (2015a) assessed peripheral whole blood for mitochondrial DNA (mtDNA) copy number and found that mean relative mtDNA copy numbers increased significantly after CCI in a pig model. These results suggest that mtDNA copy number testing may be an appropriate means to assess mitochondria bioenergetics dysfunction and to non-invasively identify presence of brain injury. Mitochondria play a pivotal role in energy metabolism in normal functioning cells, however mitochondria dysfunction is a major contributor to the secondary injury cascade after TBI (Cheng et al., 2012). TBI can lead to higher energy demands for cell re-

pair that damaged mitochondria cannot meet (Cheng et al., 2012). Excessive Ca^{2+} uptake leads to mitochondrial damage, potentiating excitotoxicity, reactive oxygen species generation, and depletion of adenosine triphosphate, which can ultimately lead to cell death (Cheng et al., 2012). A number of studies have begun to examine mitochondrial response to injury in pig TBI models to further elucidate the bioenergetic response to injury (Kilbaugh et al., 2011, 2015a, b, 2016) and to test the ability of therapeutic interventions to improve mitochondrial function (Margulies et al., 2015).

Magnetic resonance imaging in pigs

Human MRI studies have found that lesion volume is a critical predictor of outcome after TBI (Chastain et al., 2009). Given the clinical significance of MRI, it is of interest to study the time-course of lesion development and progression after injury in pigs to further elucidate TBI sequelae and to test the effectiveness of potential therapeutics. However, only a limited number of MRI studies have been performed in pig TBI models. T1-weighted, T2-weighted, and FLAIR sequences have been utilized to measure focal lesion volumes in pig CCI models (Duhaime et al., 2003; Grate et al., 2003; Rosenthal et al., 2008; Karlsson et al., 2018). Duhaime et al. (2003) and Grate et al. (2003) reported that lesion volume was found to be generally larger when measured with T2-weighted or FLAIR sequences. In addition, they noted the presence of restricted diffusion at the injury site using diffusion weighted imaging (Duhaime et al., 2003). Odland et al. (2012) measured changes in diffusion weighted imaging and generated apparent diffusion coefficient maps to quantitatively measure changes in diffusivity after CCI and found that reductive ventricular osmotherapy significantly increased apparent diffusion coefficient values compared to external ventricular drainage.

High field proton magnetic resonance spectroscopy (^{1}H-MRS) has also been used to measure neurometabolic changes as a result of TBI to assess injury severity and recovery. Initial studies utilizing ^{1}H-MRS noted a number of limitations, including hemorrhage, skull thickness, and fat content, that resulted in high variability (Duhaime et al., 2003). However, subsequent studies with increased ^{1}H-MRS resolution have demonstrated changes in neurometabolites such as N-acetylaspartate, gamma-aminobutyric acid, phosphocreatine and taurine (Tau) that could be quantitatively measured (Karlsson et al., 2018). In a DAI pig model, ^{1}H-MRS results revealed a significant decrease in N-acetylaspartate/creatine, which may reflect neuronal and axonal damage (Cecil et al., 1998; Smith et al., 1998). McGowan et al. (1999) performed magnetization transfer imaging after DAI which generates quantitative magnetization transfer ratio maps that identify regions with axonal injury. They found significant reduction of magnetization transfer ratio demonstrating the presence of diffuse axonal pathology. Functional MRI (fMRI) has also been recently tested to assess changes in the brain somatosensory cortex connectivity and recovery over time in a pig CCI model (Duhaime et al., 2006). Evidence of disruption of the somatosensory cortex after TBI was observed, however the authors stated that improved fMRI resolution is needed to more accurately and quantitatively measure functional changes longitudinally. These studies demonstrate that MRI and MRS human protocols can be utilized in pigs, and important phenomena studied in humans can now be studied and manipulated in pig models to obtain a deeper understanding of TBI pathophysiology. In addition, this also suggests that novel MRI and MRS sequences could be developed and tested in the pig and then be used in humans.

Conclusion

Despite the high prevalence of TBI in adults and young children, we are still limited in our understanding of the pathophysiology and functional responses associated with TBI and how to best treat and restore lost function. Pig TBI models provide the ability to break TBI down into more specific components, to tease apart the biggest contributors to injury progression, and to test the ability of potential treatments to mitigate injury. The gyrencephalic pig brain with its high proportion of white matter sets it apart from other small animal TBI models, particularly in regards to the susceptibility of white matter to injury after both focal and diffuse TBI. Pig TBI models will be critical in future studies to identify white matter specific recovery mechanisms that typical small animal models cannot fully recapitulate. Furthermore, the use of newly developed genetically engineered pigs with comorbidities associated with TBI, such as hypercholesterolemia (Davis et al., 2014), which may increase vascular complications after TBI, may be a useful way to tests the effectiveness of potential therapeutics. Additional studies in pigs, particularly in regards to characterizing and improving affected cognitive processes and motor impairments, are vital in moving the field towards the ultimate goal of restoring total function after TBI.

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Additional files:
Additional Table 1: *Overview of pig traumatic brain injury preclinical studies using diffuse axonal injury, controlled cortical impact, or fluid percussion injury models.*
Additional file 1: *Open peer review report 1.*

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