Development of a Minipig Model of BINT From Blast Exposure Using a Repeatable Mobile Shock Expansion Tube

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ABSTRACT

Introduction:

The Office of Naval Research (ONR) sponsored the Blast Load Assessment Sense and Test (BLAST) program to provide an approach to operationally relevant monitoring and analysis of blast exposure for optimization of service member performance and health. Of critical importance in this effort was the development of a standardized methodology for preclinical large animal studies that can reliably produce outcome measures that cannot be measured in human studies to support science-based guidelines. The primary advantage of this approach is that, because animal studies report physiological measures that correlate with human neuropathology, these data can be used to evaluate potential risks to service members by accounting for the anatomical and physiological differences between humans and large animal models. This article describes the methodology used to generate a comprehensive outcome measure dataset correlated with controlled blast exposure.

Methods and Materials:

To quantify outcomes associated with a single exposure to blast, 23 age- and weight-matched Yucatan minipigs were exposed to a single blast event generated by a large-bore, compressed gas shock tube. The peak pressure ranged from 280 to 525 kPa. After a post-exposure 72-hour observation period, the physiological response was quantified using a comprehensive set of neurological outcome measures that included neuroimaging, histology, and behavioral measures. Responses of the blast-exposed animals were compared to the sham-treated cohort to identify statistically significant and physiologically relevant differences between the two groups.

Results:

Following a single exposure, the minipigs were assessed for structural, behavioral, and cellular changes for 3 days after exposure. The following neurological changes were observed:

Structural—Using Diffusion Tensor Imaging, a statistically significant decrement ($P < .001$) in Fractional Anisotropy across the entire volume of the brain was observed when comparing the exposed group to the sham group. This finding indicates that alterations in brain tissue following exposure are not focused at a single location but instead a diffuse brain volume that can only be observed through a systematic examination of the neurological tissue.

Cellular—The histopathology results from several large white matter tract locations showed varied cellular responses from six different stains. Using standard statistical methods, results from stains such as Fluoro-Jade C and cluster of differentiation 68 in the hippocampus showed significantly higher levels of neurodegeneration and increased microglia/macrophage activation in blast-exposed subjects. However, other stains also indicated increased response, demonstrating the need for multivariate analysis with a larger dataset.

Behavioral—The behavior changes observed were typically transient; the animals' behavior returned to near baseline levels after a relatively short recovery period. Despite behavioral recovery, the presence of active neurodegenerative and inflammatory responses remained.

Conclusions:

The results of this study demonstrate that (1) a shock tube provides an effective tool for generating repeatable exposures in large animals and (2) exposure to blast overpressure can be correlated using a combination of imaging, behavioral, and histological analyses. This research demonstrates the importance of using multiple physiological indicators to track blast-induced changes in minipigs. The methodology and findings from this effort were central to developing machine-learning models to inform the development of blast exposure guidelines.

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INTRODUCTION

Between 2000 and 2017, the Department of Defense reported that 375,230 servicemen and women were diagnosed with traumatic brain injury (TBI), with 82.3% of them categorized as mild traumatic brain injuries (mTBIs).[1] With the rise of irregular warfare, blast-induced injuries have contributed to an increasingly large number of service-related TBI. Unfortunately, blast-induced neurotrauma (BINT) is likely to go undetected or unreported because of more severe co-morbidities and a lack of objective, sensitive metrics for clinical identification of such injuries.[2] In response, the Department of Defense mandated that all service members within 50 m of explosive detonation must be seen by a clinician and possibly stand down for 24 hours.[3] However, there is a lack of clinical data for blast-related events (e.g., exposure and repetition) in military populations that result in quantifiable injury and, as a result, the scientific rationale for these mandates is minimal. Ultimately, arbitrary directives such as the 50-m rule may compromise medical and operational readiness of the force.

To address these limitations, the Blast Load Assessment Sense and Test (BLAST) program undertook this study to develop methods for investigating and detecting BINT. The cornerstone is the selection of a relevant *in vivo* model that could be used to generate data relevant to human prepathological and pathological conditions, meeting the following primary criteria: (1) physiological relevance of the animal model, (2) repeatability of the primary blast wave, and (3) availability of outcome measures possibly correlated to humans. Translation of rodent blast studies to humans is limited by large differences in size, brain structure, and white matter–gray matter ratio.[4] In contrast, the authors chose Yucatan minipigs, as they have a gyrencephalic brain (similar gyral complexity as humans) and a similar craniospinal angle (relevant to the distribution of mechanical energy from blast), both of which are important determinants of human behavioral, neurochemical, and pathologic responses to brain injury.[5] Studies have used pigs for blast neurotrauma research[6–9] and collectively identified a number of neurological injuries and physiological changes associated with blast exposure; however, a comprehensive dataset that could be used to develop an injury threshold does not exist.

This manuscript describes the methods developed to investigate and detect BINT in a large animal model. To the best of our knowledge, this is the first study to investigate primary blast exposure using a repeatable, large diameter mobile shock tube and to assess biological response with neuroimaging, behavior, and histology in the minipig. This study is crucial to establishing the relationship between blast exposure parameters represented by incident blast metrics and BINT responses not possible to measure in human studies to provide science-based evidence for blast thresholds needed to protect service members.

MATERIALS AND METHODS

Mobile Shock Tube and Experimental Setup

A Friedlander-type blast wave was generated from a compressed gas-driven, mobile shock tube, designed and constructed by Applied Research Associates, Inc. (Littleton, CO) (Fig. 1A). Fabricated from 2.5-cm-thick carbon steel, the shock tube consists of high-pressure driver and driven sections, separated by a frangible diaphragm membrane of 6061-T6 aluminum alloy sheets (Aircraft Spruce, Corona, CA). The driver section of the tube houses a grate to capture fragments from the metal membrane preventing shrapnel injuries and a 15-degree expansion cone in which the animal is placed during testing. The expansion cone was necessary to allow the planar blast wave to expand and form a hemispherical wave similar to that generated by a live blast. Researchers placed each pig inside the tube to replicate a live blast scenario (Fig. 1A). Compressed helium gas generated the blast wave.

Researchers measured the incident impulse levels generated in the driven section at two different locations with piezoresistive pressure sensors (Kulite XT-190 (M) Series; Kulite Semiconductor Products, Inc., Leonia, NJ). Gauge 1 was pointed away from the animal's head to minimize recording any reflections that might occur off the head. Researchers recorded data from both pressure sensors at a 1-MHz sampling rate.

Repeatability Analysis

To minimize error in comparing the outcome data, it is critical that the pressure output from the shock tube is reproducible. Researchers verified the repeatability of the pressure–time profile through two analyses performed using gauge 1 pressure from 32 tests conducted at 345 kPa (50 psi). First, researchers compared the deviation in the peak pressure from 6 months of testing. Second, researchers applied a least mean squares approach using the least mean squares function in MATLAB to examine all 40,000 data points in each trace, including the exponential decay, and compared them to the mean impulse trace. Researchers collected pressure–time data at a sampling rate of 1 MHz with an anti-aliasing filter of 200 kHz and post-processed data with a 100 kHz Butterworth filter with the leading edge of the traces aligned to account for the variation in the membrane burst pressure.

Animal and Care Protocol

For *in vivo* blast-induced TBI characterization, intact, post-puberty, male Yucatan minipigs (Sinclair BioResources, Auxvasse, Missouri) were exposed to blast (blast $n = 16$, sham $n = 7$). The animals were an average of 26 ± 7.2 weeks old, weighing 24 ± 2.1 kg. The Virginia Tech Institutional Animal Care and Use Committee and the U.S. Army Medical Research and Materiel Command Animal Care and Use Research Office approved all protocols used in this study.

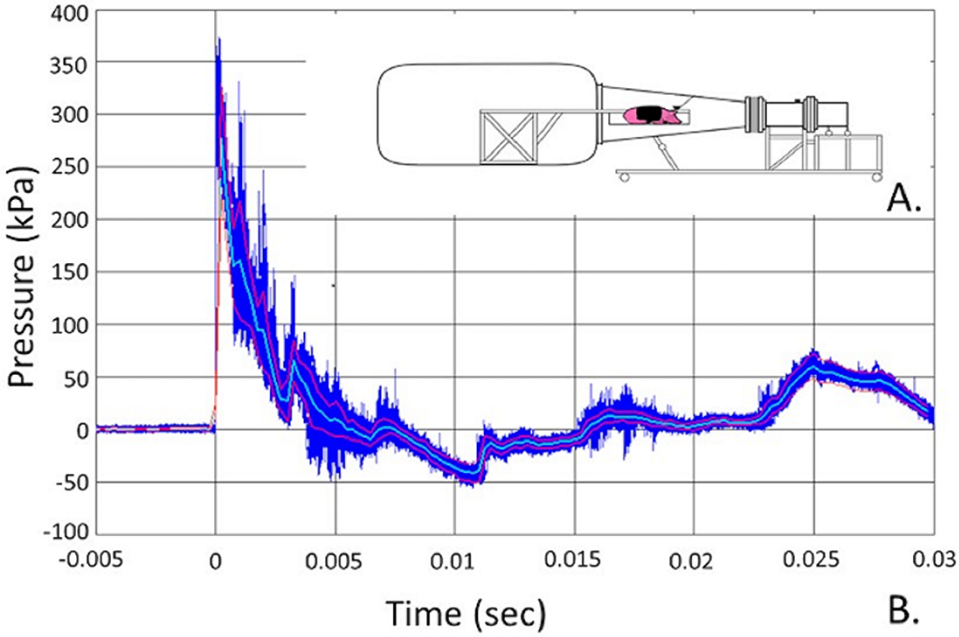

FIGURE 1. (A) Schematic of the large mobile shock tube. Subjects were placed in sternal recumbency with the head centered in the expansion cone. Impulse was measured using a piezoresistive pressure transducer mounted to a splitter plate near the subject's head (gauge 1). (B) Repeatability analysis from 32 traces at 345 kPa. The light blue and red lines show the mean value and standard deviation, respectively.

Animals were housed and cared for according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. A standard veterinary health examination and baseline neurological examination were given after arrival to determine any issues with the animals upon arrival and allow for comparison of post-exposure outcomes (48 hours) to baseline, evaluating cranial nerve functions and observing coordination, gait, and ocular damage.[10]

Magnetic Resonance Imaging Anesthesia Protocol

Before each anesthetic regime, animals were water fasted for 12 hours. Baseline magnetic resonance imaging (MRI) was performed a minimum of 6 days (11.8 average) before blast exposure. Researchers administered acepromazine (0.1-0.2 mg/kg, pre-oxygenation) 30 minutes before induction of anesthesia to facilitate manual restraint. Following pre-oxygenation, researchers induced anesthesia with isoflurane (5%) via facemask. Researchers performed endotracheal intubation, and anesthesia was maintained during the instrumentation period. Mechanical ventilation was initiated to maintain normocapnia (end tidal CO_2 35-45 mmHg). Respiratory rate, end tidal CO_2, and heart rate were continuously monitored. Anesthesia was maintained for the duration of the imaging sequence. Upon completion of imaging, pre-exposure animals returned to their housing unit once ambulatory. Researchers imaged post-exposure animals 3 days following blast testing and euthanized with sodium pentobarbital.

Blast Testing Anesthesia Protocol

On the day of blast exposure, animals were induced with isoflurane as described. However, researchers transitioned animals from isoflurane to total intravenous anesthesia after catheterizing an auricular or lateral saphenous vein with a 20-gauge 32 mm length over-the-needle catheter, so they could be ventilated, if needed. Loading doses of propofol (2 mg/kg, IV) and fentanyl (0.005 mg/kg, intravenous) were administered and isoflurane was discontinued. Researchers monitored the animals continuously for respiratory rate and heart rate. Owing to the configuration of the shock tube, the animal had to breathe while under anesthesia (without any equipment); therefore, a bolus of alfaxalone (3 mg/kg, intramuscular) was administered before transitioning to the shock tube. Alfaxalone is a neuroactive steroid that is similar to propofol in that it decreases cerebral blood flow, intracranial pressure, and the cerebral metabolic rate. In pigs, the respiratory rate, pulse oximetry, and end-tidal carbon dioxide do not significantly change following bolus administration of clinically relevant doses of alfaxalone.[7]

Researchers transported each anesthetized animal into the mobile shock tube. For all tests, the anesthetized pig was prone and the head was tilted down 60° from horizontal to prevent epistaxis (nosebleed). The body was supported by a sling that placed the head at the centerline of the cone. To minimize additional injuries, researchers used a National Institute of Justice Level II vest, goggles, and earplugs to protect the torso, eyes, and ears, respectively. Once positioned, researchers removed the endotracheal tube.

Researchers assessed anesthetic depth before blast and, if determined to be inadequate, a second bolus of alfaxalone was administered (0.5 mg/kg, IV). Anesthetized sham pigs went through the same experimental procedures but were not exposed to the blast wave. Blast-treated animals were exposed to a single blast overpressure with peak magnitudes ranging from 280 to 525 kPa.

Immediately after blast/sham exposure, researchers assessed animals for apnea and returned to the testing facility. There, researchers administered a dose of buprenorphine (0.01-0.05 mg/kg, IM), and the animals returned to their housing unit, after becoming ambulatory. Researchers monitored animals for signs of epistaxis, diarrhea, anorexia, lethargy, strabismus, and nystagmus after injury. Approximately 48 hours after blast exposure, researchers repeated neurological examinations on all animals.

Neuroimaging Data Collection

An MRI was performed with a Siemens Tim Trio 3 Tesla scanner, using three elements of an 8-channel spine array coil and supine subjects. T1-weighted anatomical images were acquired with a 3D Magnetization-Prepared Rapid Gradient-Echo (MPRAGE) sequence at 1 mm isotropic resolution and scanning parameters: TI = 900 ms, TR = 2.3 s, TE = 3 ms, FA = 9°, BW = 140 Hz/pixel, and GeneRalized Autocalibrating Partial Parallel Acquisition (GRAPPA) parallel imaging factor of 2, for an acquisition time of 11 minutes. T2 Fluid-attenuated inversion recovery (FLAIR) images were acquired with TI = 2,500 ms, TE = 96 ms, TR = 6,790 ms, with an FOV of 384 × 384 mm^2 at 2 × 2 mm^2 in-plane resolution for 30 2-mm slices with 2-mm slice gap. Researchers acquired diffusion-weighted imaging (DWI) data with a diffusion-weighted single-shot spin-echo echo planar (EPI) sequence along 64 directions with a *b*-value of 1,000 s/mm^2. Each diffusion volume was acquired as 30 2-mm slices covering the whole brain with an in-plane resolution of 2 × 2 mm^2. Additional DWI parameters include TR = 4.7 s, TE = 104 ms, BW = 1,736 Hz/pixel, and GRAPPA parallel imaging factor of 2. Researchers averaged two DWI datasets to improve SNR, with a combined scan time of 10 minutes.

Neuroimaging Data Analyses

Diffusion tensor imaging (DTI) is used to map and characterize the 3D diffusion of water as a function of spatial location within the brain. The diffusion tensor describes the magnitude, the degree of anisotropy, and the orientation of diffusion anisotropy. Researchers extracted and converted image data from the native format verified for data integrity (MRIcron&dcm2nii, version June 1, 2015, NeuroImaging Tools and Resource Collaboratory (NITRC)). The brain was then registered to the pig template[11] using the nonlinear spatial normalization function in SPM12 (https://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Researchers co-registered the three diffusion scalar maps to the pig template, allowing for comparison between subjects in a common coordinate space. Following transformation, researchers masked the subject's diffusion scalar maps using the template to limit all statistical analyses to voxels in the brain. The DWI was then prepared for further steps by calculating the B-Matrix from gradient tables, correcting for motion, eddy current, and B_0 susceptibility induced EPI distortions. Diffusion tensor and subsequent diffusion scalars were calculated from the DWI values (TORTOISE's DIFF_PREP & DIFF_CALC, v. 2.5.1, National Institutions of Health Pediatric Neuroimaging Diffusion Tensor MRI Center)[12] and applied to the following formulae to return the diffusion scalar maps utilized in this study:

$$\text{Fractional anisotropy (FA)} = \sqrt{1/2} * \frac{\sqrt{(\lambda_1-\lambda_2)^2+(\lambda_2-\lambda_3)^2+(\lambda_3-\lambda_1)^2}}{\sqrt{\lambda_1^2+\lambda_2^2+\lambda_3^2}}$$

To map DTI effects, researchers computed two statistical voxel-by-voxel (VbV) tests using SPM12. The first was a paired *t*-test where the post-treatment scan was paired with the pre-treatment scan, with contrasts of Post-Scan FA > Pre-Scan FA and Pre-Scan FA > Post-Scan FA. Researchers ran the test on the sham subjects and experimental subjects independently of one another. The other test was a multiple regression of experimental subjects and experimental subjects with sham subjects. The contrasts employed were a negative or positive correlation with increasing blast impulse.

In addition to the SPM-based VbV tests, the authors calculated a composite statistic over the entire brain. Specifically, a total change score (TCS) was calculated for each subject by summing the total FA post treatment and subtracting the total FA before treatment, using the formula below:

$$\text{TCS} = \sum^{s_x,s_y,s_z} \left(\text{Д}_{(x,y,z),Post} - \text{Д}_{(x,y,z),Pre}\right)$$

The authors used a Python script with R subroutines to quantify positive FA values and calculated group differences using a *t*-test.

Human Approach Test

An in-pen, human approach test (HAT) was developed using methods described previously.[9,13] This assessment quantifies fear and motivation,[14] which underlie many affective states including hypervigilance, depression, anhedonia, etc. measured clinically in humans. The same experimenter performed the HAT 3 days pre- and 3 days post-blast exposure ($n = 6$ sham and $n = 12$ blast). The initial group of animals ($n = 5$) did not undergo HAT testing, reducing total animals for HAT analyses.

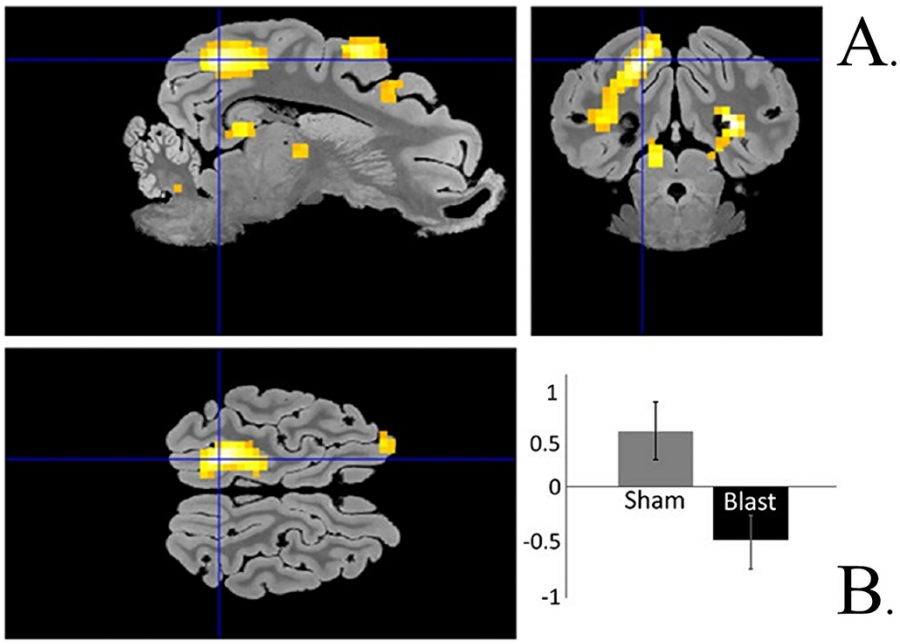

FIGURE 2. (A) The voxel-by-voxel (VbV) analysis, highlighted with yellow pixels, reflected the widespread nature of the localized differences found using the Whole Brain Approach. (B) Normalized total change score in fractional anisotropy (FA) was significantly lower in the blast group ($P < .001$).

To administer the test, the experimenter approached the pen and stood in front of the pen for a minimum of 180 seconds. Tests were recorded using a GoPro Hero3+ (San Mateo, CA) or an overhead video system (GeoVision GV-1480 16-Channel PC DVR video surveillance system; Points North Surveillance systems, Auburn, ME). Researchers coded videos using specialized software (Noldus Observer XT 11.0, Leesburg, VA) by one trained, blinded observer.

Videos were timestamped mutually exclusive states at a playback speed of 15 fps. Three ethograms were used: (1) spatial behaviors in relationship to human (close, middle, and far); (2) head-orientation (facing the human or turned away); and (3) exploratory behaviors (rubbing, sniffing, licking, and rooting). The durations for behaviors were combined in a weighted approach index, as previously described.[13] The repeated approach index data were analyzed using a linear, mixed model with the fixed effects of time, treatment, and treatment × time by restricted maximum likelihood analysis of variance using the MIXED SAS procedure (v. 9.2, SAS Inst. Inc., Cary, NC, USA). The authors used compound symmetry covariance structure for within-subject measurement and heterogeneity of variance for all models. The authors tested data for normality of residuals by evaluating the Shapiro–Wilk statistic using the SAS's UNIVARIATE procedure. The HAT measures were not normally distributed; therefore, they were log-transformed before mixed model analysis. Pairwise comparisons were performed among treatments at each time using a sliced-effect multiple comparison approach and within each treatment across time using a Tukey–Kramer adjustment to control the family-wise type-1 error. Least squares means (± SEM) are reported throughout. A treatment difference of P-value < .05 was considered statistically significant.

Immunohistochemical Analysis

After the 3 days post-blast MRI, researchers used transcardial perfusion to flush the vasculature with heparinized saline, and 4% paraformaldehyde as brain fixative. Researchers administered Beuthanasia-D to euthanize the animals with an overdose of sodium pentobarbital. After perfusion, researchers removed the brain from the skull and stored it in fixative for 7 days. Once paraffin blocks were cut into 6-μm coronal sections, the hippocampus was evaluated for damage in two adjacent sections in each hemisphere. Tissue sections of the hippocampus were stained with Fluoro-Jade C (FJC) to identify neurodegeneration,[15] cluster of differentiation 68 (CD68)[16] to determine microglial/macrophage activation indicating neuroinflammation, and GFAP to measure astrocyte reactivity.[17] In other white matter tracts, researchers also examined impaired axonal transport with Beta Amyloid Precursor Protein[18] and vasoderegulation with Alpha-Smooth Muscle Actin (α-SMA)[19] in addition to astrocyte reactivity and neuroinflammation.

For this pilot study, JMP (Version: Pro 12; Cary, NC, USA) was used for all statistical analyses. The authors used residual analysis to exclude outliers, and Student's t-tests with P-values of less than .05 were considered statistically significant. Results were compared between exposed and sham groups for each outcome measure, independent of other variables, to search for the clearest responses when exposure is well-controlled in a laboratory animal model.

RESULTS

Repeatability Analysis

The authors performed two analyses on the 32 tests in the 344 kPa (50 psi) group. The first analysis showed the group had a peak pressure of 347 ± 14 kPa (50.3 ± 2.0 psi) as illustrated in Figure 1B, suggesting the traces are statistically similar within the group. Second, the authors examined the exponential decays for all traces. The analysis across all data points showed a correlation of 0.95 ± 0.04. These two analyses demonstrate that both the peak pressure and the shape of the exponential decay of the impulse were repeatable with little variation.

Neuroimaging

Analysis of T2 images did not show any observable changes after exposure to blast overpressures ranging from 280 to 525 kPa. Evaluation of the DTI results revealed structural changes, such as those shown in Figure 2A, when analyzed via two independent approaches: (1) focal changes in specific areas and (2) global changes. Some affected anatomical regions included the corpus callosum, temporal poles, superior cerebellar peduncle, and posterior cerebellum. In contrast, analysis of global changes showed a stronger relationship across all subgroups when comparing blast-exposed to sham groups. The TCS showed a statistically significant decrement ($P < .001$) in FA across the entire volume of the brain when comparing the exposed group to the sham group (Fig. 2B).

Neurological and Behavioral Tests

The standard veterinarian examinations did not show any abnormalities between before-blast and 48 hours post-exposure, nor did it show any significant differences between sham and blast animals. For behavioral assessment, researchers conducted an in-pen HAT, and the authors used the behavior durations to calculate an approach index.

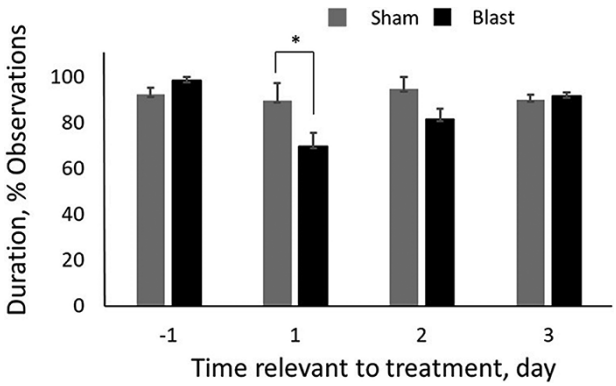

FIGURE 3. Behavioral responses to human approach test (HAT) after a single blast exposure in minipigs, showing the approach index of sham ($n = 6$) and blast-exposed pigs ($n = 12$) relative to treatment day (0). The approach index calculation placed the duration of active, approach-related behaviors on a scale of 0-100. The error bars represent the standard error means. *LS-means differ between treatments $P < .05$ at day 1.

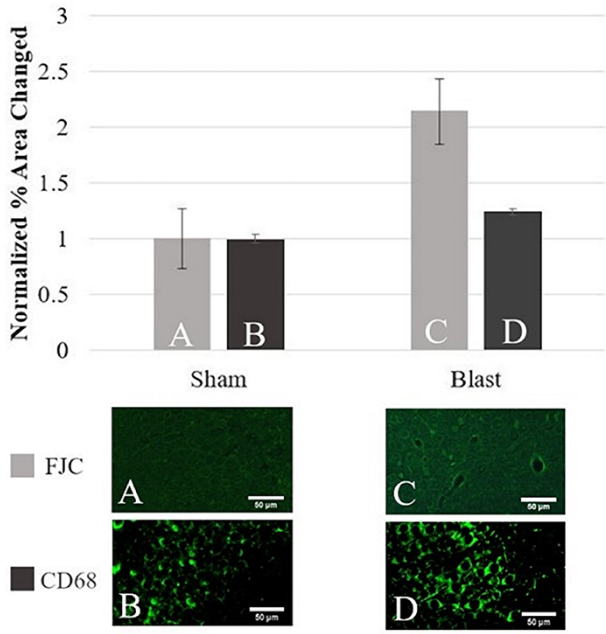

FIGURE 4. Average Fluoro-Jade C (FJC)-positive neurons in the dentate gyrus (DG) of the hippocampi of blasted animals ($N = 16$) compared to shams ($N = 7$) and representative images in the hippocampus of a sham animal (A) and blast animal (C) with a P-value < .01. Percentage cross-sectional area exhibiting cluster of differentiation 68 (CD68) immunofluorescence in the DG region of the hippocampi of blast animals ($N = 16$) compared to sham animals ($N = 7$), and representative images of a sham animal (B) and a blast animal (D) with a P-value < .01.

Before exposure, all subjects had approach indexes in the 85-100% range (Fig. 3). Sham-treated subjects' approach indexes were constant throughout the experiment ($P > .10$). However, blast-treated subjects had lower approach indexes than Sham pigs the day after treatment ($P < .05$). The within-the-blast-treatment subjects' approach indexes show an incremental increase after day 1 but did not return to their baseline index ($P < .05$; Fig. 3).

Neurodegeneration and Inflammation

Among the nine combinations of white matter tracts and staining methods observed, more than half showed statistically increased responses when analyzed with the standard statistical methods described. However, FJC and CD68 in the hippocampus showed the strongest response in this dataset and are discussed in more detail below.

The FJC results of dentate gyrus (DG) hippocampal neurons showed a significant increase in neurodegeneration in animals in the blast group compared to the sham group ($P < .01$) at 3 days after blast. Figure 4A and C depict the average FJC-positive neurons and representative images in sham

animals compared to blast animals. The CD68 immunoreactivity in the DG of the hippocampus measured neuroinflammation via microglia/macrophage activation levels. The blast animals showed significantly greater CD68 immunoreactivity compared to sham animals ($P < .01$; Fig. 4B, D).

DISCUSSION AND CONCLUSIONS

To ensure that blast pressure was a variable that could be controlled, researchers designed and developed a mobile shock tube that produced identical shock waves regardless of testing site. The authors found that our mobile shock tube not only produced similar peak pressures, but it also produced similar exponential decay of the impulses. The production of these features is important because both pressure magnitude and duration play a role in tolerance to BINT are should be considered in further analyses of outcome using this experimental model.[20] Moreover, the range of blast pressures that this mobile shock tube can produce was well within the range of real-world operational exposures (including the 50 psi in this study).

Neuroimaging has played an important role in detecting, tracking, and managing patients with TBI in clinical settings due to the technique's ability to detect changes across the volume of the brain, which is important for detecting the global effect of blast on the brain. The authors used DTI because it is one of the most sensitive neuroimaging modalities for quantitative detection of white matter integrity and diffuse axonal injury after brain injuries. Fractional anisotropy (FA), a measure of asymmetric diffusion, has been used to identify changes in patients with TBI[21] as well as in *in vivo* experimental studies.[22] In fact, clinical studies of blast-exposed veterans showed significant changes in FA compared to controls.[23] Additionally, alterations in subcortical connections correlate with the overall neuropsychological function[24] as well as changes in learning and memory performance.[25]

In the current study, the significant reductions in whole-brain FA in single blast-exposed animals (as compared to the shams) indicate that the blast-exposed animals sustained widespread white matter track disruptions detectable at 3 days post-exposure. This finding agrees with a recent clinical study where mTBI patients showed significantly lower FA in 88% of the brain regions examined.[26] This clinical and preclinical evidence suggests that the current Department of Defense guideline of a 24-hour stand-down period for soldiers within 50 m of a blast event may need further refinement.[27]

Finding significant changes in anatomically specific regions using DTI can be challenging in BINT patients. Combining evaluation of global changes with the delineation of region-specific changes significantly increased the sensitivity of our approach. Interestingly, DTI with global and regional FA measurements in patients with TBI have been shown to correlate positively with neuropsychological test scores and observed neurological deficits, further underscoring the utility of this approach.

However, a single blast exposure in pigs did not produce detectable differences between sham and blast-exposed pigs using a standard veterinarian wellness examination. Unfortunately, subclinical injury is difficult to detect and measure using qualitative examinations because individual animal temperament may confound the expression of sickness or injury behaviors.[9] For example, many pigs will not provide "honest-signaling" if their environment is perceived as a threat. In contrast, healthy subjects will engage in exploratory behaviors.

Consequently, the authors selected the least invasive behavioral methodologies available to evaluate the responses of individual pigs in a state of subclinical injury, noting that noninvasive, ethological assessment tests have been used to observe changes in behaviors related to appetitive vs. avoidance responses with a limited experimental timeline.[8,13] Using a modified HAT, researchers were able to quantify changes in subtle behaviors. Blast-treated subjects never returned to their baseline measures, indicating that they were still in subclinical states at 3 days post blast. Overall, the HAT results showed that this in-pen test is sensitive enough to detect subclinical changes in pigs in the absence of other detectable differences in a standard neurological examination. The authors posit that sensitive ethological tests such as HAT could be useful for detecting pre-pathological changes in individuals with BINT for diagnosis and recovery.

The examination of cellular changes associated with a single blast exposure in pigs showed significantly higher levels of neurodegeneration and increased microglia/macrophage activation in the hippocampi when compared to sham-treated subjects. The hippocampus is an area of the brain known to play critical roles in learning, memory, emotion,[28] and fear/anxiety.[29] It has frequently been identified as a region of interest following blast-induced TBI because symptomatic hallmarks in patients include elevated anxiety and learning and memory impairments.[30,31] While there are other brain regions affected by exposure to blast, the hippocampus remains a consistent area affected by even mTBI.[32–34] However, neuroinflammation can be more difficult to interpret as the inflammatory response after blast exposure can be either detrimental or reparative.[35,36] Future studies that focus on the time course and nature of this inflammation could elucidate the role of neuroinflammation in cellular degeneration and innate repair mechanisms after BINT.

Other measures of physiological change also showed signs of response using histochemical methods. How these responses (such as vasoderegulation) relate to the neuroinflammatory response and accompanying neurodegeneration remains to be seen. This pilot study demonstrates that a single physiological outcome measure is unlikely to capture the full effects of blast exposure on the brain and that multiple cellular mechanisms are needed to identify the underlying causes of observed outcomes. Moreover, multivariate analyses combining histopathological responses with observed structural and behavioral responses have given even more insight into how

the changes observed in this pilot study relate to one another.[20] Such analyses could yield valuable insight into the mechanisms of BINT and are crucial to determining safe exposure levels.

Despite the prevalence of survivable mTBI in warfighters, there are no clear guidelines for the diagnosis of BINT. In the absence of BINT neuropathology in humans, animal models can serve as a valuable source of data on the relationship between blast exposure levels and the risk of brain injury. This manuscript establishes a standard for a preclinical large animal model and a methodology to produce BINT reliably, with a series of outcome measures that may be correlated with neuropathology in humans suffering from BINT. This methodology allows researchers to examine BINT in a controlled laboratory setting, providing a basis for establishing a dose–response relationship between blast exposure and outcome in ways that are not possible with human subjects. Using incident pressure as a standardized environmental dose, further analyses built on this methodology were used to developed thresholds for blast exposure[20] and translate the dose–response relationship to human-relevant terms,[37] establishing science-based guidelines as part of the BLAST program.[38]

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CONFLICT OF INTEREST STATEMENT

No competing financial interests exist.

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