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TITLE:

Low-intensity blast wave model for preclinical assessment of closed-head mild traumatic brain injury in rodents

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KEYWORDS:

mild traumatic brain injury, blast-induced, coup and contrecoup injury, rotational forces, concussion, compressed gas shock tube, numeric pressure sensor, bench-top setup, neuroinflammation, mouse, rat.

SUMMARY:

We present here a protocol of a blast wave model for rodents to investigate neurobiological and pathophysiological effects of mild to moderate traumatic brain injury. We established a gas-driven, bench-top setup equipped with pressure sensors allowing for reliable and reproducible generation of blast-induced mild to moderate traumatic brain injury.

ABSTRACT:

Traumatic brain injury (TBI) is a large-scale public health problem. Mild TBI is the most prevalent form of neurotrauma and accounts for a large number of medical visits in the United States. There are currently no FDA-approved treatments available for TBI. The increased incidence of military-related, blast-induced TBI further accentuates the urgent need for effective TBI treatments. Therefore, new preclinical TBI animal models that recapitulate aspects of human blast-related TBI will greatly advance the research efforts into the neurobiological and pathophysiological processes underlying mild to moderate TBI as well as the development of novel therapeutic strategies for TBI.

Here we present a reliable, reproducible model for the investigation of the molecular, cellular, and behavioral effects of mild to moderate blast-induced TBI. We describe a step-by-step protocol for closed-head, blast-induced mild TBI in rodents using a bench-top setup consisting of a gas-driven shock tube equipped with piezoelectric pressure sensors to ensure consistent test conditions. The benefits of the setup that we have established are its relative low-cost, ease of installation, ease of use and high-throughput capacity. Further advantages of this non-invasive TBI model include the scalability of the blast peak overpressure and the generation of controlled reproducible outcomes. The reproducibility and relevance of this TBI model has been evaluated in a number of downstream applications, including neurobiological, neuropathological, neurophysiological and behavioral analyses, supporting the use of this model for the characterization of processes underlying the etiology of mild to moderate TBI

INTRODUCTION:

Traumatic brain injury (TBI) accounts for more than two million hospital visits each year in the United States alone. Mild TBI commonly resulting from car accidents, sporting events, or falls represent approximately 80% of all TBI cases[1]. Mild TBI is considered the 'silent disease' as patients often experience no overt symptoms in the days and months following the initial insult, but can develop serious TBI-related complications later in life[2]. Moreover, blast-induced mild TBI is prevalent among military service-members, and has been associated with chronic CNS dysfunction[3-6]. Due to the rising incidence of blast-related mild TBI[7,8], preclinical modeling of neurobiological and pathophysiological processes associated with mild TBI has thus become a focus in the development of novel therapeutic interventions for TBI.

Historically, TBI research has primarily focused on severe forms of neurotrauma, despite the relatively lower number of severe human TBI cases. Preclinical rodent models for severe human TBI have been developed, including the controlled cortical impact (CCI)[9,10] and fluid percussion injury (FPI)[11] models, which are both well established to produce reliable pathophysiological effects[12,13]. These models have laid the groundwork for what is known today about neuroinflammation, neurodegeneration, and neuronal repair in TBI. Although considerable knowledge of the pathophysiology of TBI has been developed, there are currently no effective, FDA-approved treatments available for TBI.

More recently, the focus of TBI research has been broadened to include a wider spectrum of TBI-related pathologies with the ultimate goal of developing effective therapeutic interventions. Nevertheless, few preclinical models for mild TBI have been established that

have shown measurable effects, and only a small number of studies have investigated the mild TBI spectrum[2,14,15]. As mild TBI accounts for the large majority of all TBI cases, reliable models of mild TBI are urgently needed to facilitate research into the etiology and neuropathophysiology of the human condition, in order to develop novel therapeutic strategies.

In conjunction with biomedical engineers and aerospace physicists, we have established a scalable, closed-head blast wave model for mild to moderate TBI. This preclinical rodent model has been specifically developed to investigate the effects of force dynamics, including blast waves and acceleration/deceleration movement, that are associated with human mild TBI obtained in military combat, sporting events, car accidents, and falls. As blast waves correlate with the force dynamics that cause mild TBI in humans, this model was designed to produce a consistent Friedlander waveform with an impulse, which is measured as pounds per square inch (psi)*millisecond (ms). The impulse level is scaled to fall below defined lung lethality curves for mice and rats in order to conduct preclinical investigations[16-18]. In addition, this model allows for investigation of coup and contrecoup injury due to rapid rotational forces of the animal's head. This kind of injury is inherent to several types of clinical TBI presentations, including those observed in both military and civilian populations. Therefore, this versatile model fits a need that encompasses multiple clinical presentations of TBI.

The preclinical model presented here produces reliable and reproducible pathophysiological changes associated with clinical mild TBI as demonstrated by a number of prior studies[17,19-23]. Studies with this model showed that rats subjected to a low-intensity blast wave exhibited neuroinflammation, axonal injury, microvascular damage, biochemical changes related to neuronal injury and deficits in short-term plasticity and synaptic excitability[19]. However, this mild TBI model did not induce any macroscopic neuropathological changes, including tissue damage, hemorrhage, hematoma and contusion[19] that have been commonly observed in studies using moderate to severe invasive TBI models[10,24]. Previous research[19,21-23] has shown that this preclinical model can be used to characterize neurobiological and pathophysiological processes underlying the etiology of mild and moderate TBI[17,19-23]. This model also permits for testing of new therapeutic compounds and strategies, as well as the identification of novel, suitable targets for the development of effective TBI interventions[19,21-23].

This model was developed to investigate effects induced by blast waves as well as rapid rotational forces on molecular, cellular and behavioral outcomes in rodents. Analogous to the blast wave model presented here, a number of preclinical models has been developed that attempt to recapitulate mild to moderate TBI using gas-driven overpressure waves[2,14,17,25-28]. Some of the limitations of other models include: the animal is fixed to a wire-mesh gurney and the head is immobilized upon impact; the peripheral organs are exposed to the wave in addition to the brain, which creates the confounding variables of polytrauma; and the models are large and stationary, which limits changing and adapting critical parameters to better model conditions reminiscent of human TBI.

The benefits of this bench-top, gas-driven shock tube setup are its relative low-cost for acquisition and running expenses, as well as ease of installation and use. Furthermore, the

setup allows for high-throughput operation and generation of controlled reproducible blast waves and in vivo outcomes in both mice and rats. In order to control for consistent test conditions (i.e., constant blast wave and overpressure) the setup is equipped with pressure sensors. The advantages of this model for TBI include scalability of the injury severity and that mild TBI is induced using a non-invasive, closed-head procedure. Peak overpressure and subsequent brain injury increase with thicker polyester membranes in a consistent scalable manner[17]. The ability to scale TBI severity through membrane thickness is a useful tool to determine the level, at which specific outcome measures (e.g., neuroinflammation) become evident. Providing protective shielding for the peripheral organs, also allows focused investigation into mild TBI mechanisms by avoiding or reducing confounding variables of systemic injury, such as lung- or thoracic injury. Moreover, this setup allows selecting the direction, by which the blast wave strikes/penetrates the head (i.e., head-on, side, top or underneath) and therefore different types of TBI-inducing insults can be investigated. The standard procedure to induce mild to moderate TBI described here employs side exposure to evaluate the effects of blast wave injury in combination with coup and contrecoup injury due to rapid rotational forces. Furthermore, in order to investigate exclusively blast-induced injury, top down blast wave exposure can be employed in this model.

PROTOCOL:

The protocol follows the animal care guidelines of the University of Cincinnati and West Virginia University. All procedures involving animals were approved by the *Institutional Animal Care and Use Committees* (IACUC), and were performed according to the principles of the *Guide for the Care and Use of Laboratory Animals*.

1. Installation of the blast TBI setup

1.1. Acquire all the working parts that are required for the setup, including: shock tube consisting of steel driven- and driver section, polyester membrane, securing bolts, pressure sensors, polyvinyl chloride (PVC) pipe shield to protect peripheral organs, 9.53 mm high pressure hydraulic line and quick connect male and female attachments, high flow gas regulator and a gas cylinder with wall-mount bracket (see **Figure 1A,B** and **Table of Materials**).

NOTE: The specifications of the driven and driver section used here (see **Figure 2** and **Table of Materials**) have been established to produce a consistent short-duration scaled blast wave (see **Figure 3C**,**D**) to induce mild to moderate TBI in mice. For this purpose, a taper-designed (6° taper) short driver section was selected. The length and diameter of the driven and driver sections can be modified to specifically research blast wave[29-32], compression wave[18], or shock wave dynamics[33]. For experiments with rats, the dimensions of the shock tube need to be adapted to yield comparable forces according to retain pertinent body scaling parameters[17] (see **Table of Materials**).

1.2. Install the individual working parts of the setup on machine slide tables that are fixed on a stable, easy to clean surface (preferably stainless steel for use in rodents) in laboratory space approved for animal experiments.

NOTE: The blast wave experiments produce a considerable level of noise; therefore choose a location within sound absorbing laboratory space, where noise will not interfere with other experiments/laboratory groups.

1.2.1. Fix the PVC pipe shield perpendicular to the shock tube setup so that the body of the rodent will be fully covered and only the head protrudes.

NOTE: For the standard procedure to induce mild to moderate TBI described here, the center of the head is located 5 cm from the end of the driven section for mice.

1.2.2. Wall mount gas cylinder in close proximity to setup in accordance with OSHA and all other pertinent safety regulations.

NOTE: Compressed air, helium or nitrogen gas are commonly used to generate the blast waves in rodent shock tube models. All data presented here has been generated using helium, as this gas produces higher overpressure over a shorter duration,[34] allowing for appropriate scaling for murine subjects .

2. Evaluation of the setup and blast wave properties using pressure sensor recordings.

2.1. Prepare the shock tube.

2.1.1. Carefully cut the polyester membrane without bending and producing fissures, in order to ensure consistent rupture.

2.1.2. Insert the membrane between the driven and driver sections. Secure the sections by tightening connecting bolts.

2.1.3. Check that the system is airtight and the membrane is tightly fixed between driver and driven sections.

2.1.4. Connect the gas tank via a 9.53 mm high pressure hydraulic hose and quick connect attachments to the shock tube

NOTE: Driver and driven sections are machined to precise tolerances in order to afford a complete seal of membrane between sections. This allows for no gas leakage and precludes the use of any form of gasket/o-ring material and allows for greater consistency in generated waveform.

2.2. Install the pressure sensors for monitoring the blast waves (see **Figure 1C**).

2.2.1. Position one pressure sensor in the head placement area, and three sensors at the exit of the shock tube (see **Figure 1C** and **2**).

2.2.2. Initiate recording from pressure sensors, just prior to blast wave execution. Record the pressure wave data at 500,000 frames per second using a sensor signal conditioner and data acquisition board (see **Table of Materials**).

NOTE: Wear OHSA-approved earmuffs to ensure adequate hearing protection.

2.2.3. Open the main valve of the compressed gas tank fully to allow the gas flow to produce a sudden, rapid pressure spike.

NOTE: The gas overpressure ruptures the polyester membrane to release a shock wave that transitions into a compression wave within the driven section and exits the tube in the direction of the head placement area.

2.2.4. Turn off the gas flow immediately after the procedure.

NOTE: The setup can be equipped with a spring return valve to automatically and rapidly stop the gas flow.

2.2.5. Analyze the pressure wave recordings using custom written computer program to determine peak overpressure and graph data. Data can be graphed with each sensor individually or overlaid on one another to demonstrate planarity of the wave generated (see **Figure 3C,D**).

NOTE: The analysis can technically be done using more readily available software, but due to the large data sets, these programs have long delays in generating plots.

2.3. Establish experimental conditions that are adequate for the aim of the designated TBI study, and confirm that the model produces a consistent blast wave with a peak over-pressure, duration, and impulse measurement comparable to a Friedlander wave (see **Figure 3**). Verify these parameters using the aforementioned computer software.

2.3.1. Calibrate the setup by repeating steps 2.1.1. to 2.2.5. and use the pressure wave recordings to determine whether the setup needs adjustment (for representative data see **Figure 3**).

2.3.2. Modify the setup (if needed).

NOTE: The blast wave properties can be adjusted by minor modifications of the setup. For example, the distance of the head to the end of the driven section impacts the blast wave force at the level of the head. The thickness of the polyester membrane determines the level of peak

overpressure, with thicker membranes increasing peak levels (see **Figure 3A,B**). Additionally, the setup allows selecting the direction by which the blast wave strikes/penetrates the head (i.e., head-on, side, top or underneath) and therefore different aspects can be investigated, such as blast wave injury alone or in combination with coup and contrecoup injury due to rapid rotational forces.

2.3.3. Repeat steps 2.1.1 to 2.2.4 to establish desired blast wave properties (if needed) and control for reproducibility.

2.3.4. Repeat steps 2.1.1 to 2.2.4 with polyester membranes of different thickness to evaluate scalability of the setup (for representative data see **Figure 3A,B**).

3. Preparation of experimental setup and induction of mild TBI in rodents

NOTE: Transfer rodents to holding area 30 min to 1 h prior to start of TBI experiments to acclimatize. Select holding area that is minimally affected by noise of the procedure.

3.1. Prepare all materials required for the experiment and check setup for proper installation (e.g., adjust parameters according to aim of study) (~5 – 10 minutes).

NOTE: Injury severity can be adjusted by selecting the thickness of the polyester membrane. Based on our studies, a membrane thickness of 25.4 to 102 μm is utilized for mild to moderate TBI in mice[35]. We have previously utilized membranes with a thickness of 76.2 to 127 μm to produce mild to moderate TBI in rats[19].

3.1.1. Carefully cut the polyester membrane, insert it between the driven and driver sections and secure by tightening the connecting bolts.

3.1.2. Connect the gas tank to the shock tube through the use of quick-release fittings. Ensure that the membrane is tightly fixed between driver and driven sections.

3.1.3. Place three pressure sensors at the exit of the shock tube, 120° apart, to monitor the blast wave properties during the TBI induction as described in step 2.2.2 and 2.2.5.

3.1.4. Ensure distance from end of shock tube apparatus is correct for each respective subject using installed micrometer. Keep the positioning of the rodent's head (i.e., position, distance) constant within studies to allow for consistent injury evaluation.

NOTE: As stated in 1.2.1., different types of injury can be induced by selecting the direction, in which the blast wave impacts the head. For the procedure to induce mild to moderate TBI described here, the body is placed perpendicular to the shock tube that the blast wave impacts the side of the head. In this setting, the head is allowed free mobility and hence is exposed to the blast wave and rapid rotational forces allowing the generation of coup and contrecoup effects.

3.1.5. Initiate the recording from the pressure sensors using the graphical user interface (GUI) of the software.

3.2. Anesthesia and positioning of rodents in setup

3.2.1. Transfer rodents from holding room and induce anesthesia with 4% isoflurane in oxygen and maintain with 2% isoflurane in oxygen to reduce distress and pain.

NOTE: Make sure the animal is non-responsive to toe or tail pinch prior to proceeding. Make sure the induction of anesthesia is consistent for all experimental animals, including sham controls. This procedure requires a low-level and short duration of anesthesia.

3.2.2. Place the fully anesthetized rodent in the PVC pipe shield with cushioning to protect peripheral organs from the blast wave.

NOTE: Control subjects are anesthetized and placed in proximity to the setup, but are not directly subjected to the blast wave. Ensure that the controls are subjected to the noise generated by the shock tube.

3.2.3. Place the head of the rodent within the head placement area and support it from below, either by a support built directly into the shielding apparatus or a gauze pad. Determine the head alignment according to each individual rodent's anatomy, with the occipital condyle aligned with the edge of the protective shielding.

NOTE: Avoid directing the pressure wave directly towards the brainstem to decrease mortality. Injury to the respiratory center of the brainstem and the cervical spinal cord is known to contribute to breathing abnormalities and even death in rodent models of TBI[36-38].

3.3. Exposure of rodents to blast wave.

3.3.1. Rapidly open the main valve of the compressed gas tank to produce a pressure spike that ruptures the membrane and produce a loud explosion that confirms the generation of a pressure wave. The membrane will be visually ruptured when removed after the experiment.

NOTE: A high-speed camera can be used to capture the coup and contrecoup effects of the rotational acceleration experienced by the rodent for further analysis.

3.3.2. Turn off the gas flow immediately after hearing the explosion.

3.3. Recovery from blast wave exposure

3.3.1. After blast wave exposure, remove the rodent from the apparatus and place on flat surface directly adjacent to shock tube on their side.

3.3.2. Monitor subjects to determine righting reflex time (RRT). Use a stopwatch to record time from blast wave exposure until they regain inherent righting reflex. (see **Figure 4A**).

3.3.3. As soon as subjects regain their righting reflex, place them in their respective home cage where they are monitored for adverse reactions (i.e., seizures, difficulty breathing, bleeding from a bodily orifice) for the next 24 h.

3.3.4. After the initial monitoring period, subjects can be analyzed using various biochemical, neuropathological, neurophysiological, and behavioral assays of researcher's choosing (see below).

3.4. Prepare setup and room for next experiment.

3.4.1. Clean setup with detergent to remove odor.

4. Downstream applications for rodents exposed to blast wave/rotational forces and controls

NOTE: In previous studies, the effects of mild to moderate TBI at various time points after exposure to a blast wave and rotational forces were assessed in rodents using downstream applications, including biochemical, neuropathological, neurophysiological, and behavioral analyses[19].

4.1. Biochemical analysis

4.1.1. At defined experimental time points (hours to days post-mild TBI), harvest tissue (e.g., brain, blood) for biochemical analysis using standard protocols as described[19].

4.1.2 Use tissue for biochemical analysis (i.e., immunoblotting, Elisa, etc.) to assess the effect of mild TBI on neurobiological and pathophysiological processes.

4.2. Neuropathological analysis

4.2.1. At defined experimental time points (hours to days post-mild TBI), perfuse rodents transcardially with saline solution followed by 4% paraformaldehyde solution to fix tissue as described[19].

NOTE: Some applications are not compatible with paraformaldehyde fixation (e.g., silver staining, some antibodies for immunohistochemistry).

4.2.2. Use perfused, fixed tissue for anatomical, histological and molecular analyses to assess neuropathological changes associated with mild TBI, including neuroinflammation, neurodegeneration, and neurochemical changes as described[19].

4.3. Neurophysiological analysis in brain slices

4.3.1. At defined experimental time points (hours to days post-mild TBI), sacrifice rodents by decapitation, remove brain and prepare brain slices as described[19].

4.3.2. Perform electrophysiological recordings as described[19] to assess the effect of mild TBI on basal synaptic properties and synaptic plasticity.

4.4. Behavioral analysis

4.4.1 At defined experimental time points (hours to days post-mild TBI), evaluate behavioral performance, including motor function (e.g., open field, rotarod, locomotor activity; see **Figure 4D**) and learning & memory (e.g., fear conditioning, Barnes maze, Morris water maze).

REPRESENTATIVE RESULTS:

The scalability of the blast wave setup was tested using three different membrane thicknesses, 25.4, 50.8 and 76.2 μm. Peak pressure levels were assessed at the head placement area and the exit of the shock tube apparatus using piezoelectric pressure sensors (see **Figure 1** & **Figure 2**). Peak pressures increase in concordance with membrane thickness at both sensor locations (**Figure 3A,B**), demonstrating that the peak pressure is scalable in nature. This property of the setup can be exploited to calibrate the system and assess its scalability as described in step 2.3.

In order to evaluate effects of blast-induced TBI in vivo, adult, 3-month-old, male, wild-type C57Bl/6J mice were exposed to blast waves produced by this setup (**Figure 1 & 2**) using the protocol described here. First, the effects of blast waves produced with two different membrane thicknesses (50.8 and 76.2 μm) or sham treatment on righting reflex time (RRT) were assessed (**Figure 4A**). The latency of the mice to fully right themselves (4 paws on the ground) after anesthesia is determined here as RRT. The mice were anesthetized using isoflurane (consistent, short and mild anesthesia) and then underwent TBI induction or sham treatment. Immediately following injury, mice were allowed to recover and time to regain righting reflex was recorded. Mice that were exposed to a blast wave produced with the 76.2 μm membrane exhibited a significant increase in RRT as compared to sham controls that underwent the same anesthesia procedure (**Figure 4A**), suggesting that this blast wave induces loss of consciousness. In contrast, mice exposed to a blast wave from the 50.8 μm membrane exhibit no significant increases in RRT (**Figure 4A**), indicative of mild form of TBI. Rupture of a standard 76.2 μm polyester membrane results in the rapid generation of a short duration blast wave of approximately 160 psi of overpressure (**Figure 3C**), which the left side of the subject's cranium is exposed to during the experimental procedure.

The short-term physiological effects occurring after the exposure to blast wave and rotational forces in rodents are currently not well characterized. To delineate the acute effects of blast wave exposure and rotational forces from this model, we assessed core body temperature regulation and body weight. The temperature and body weight of adult, 3-month-old, male

wild-type C57Bl/6J mice were recorded following TBI induction. Baseline core body temperature and body weight were recorded in the mice prior to TBI procedure or sham treatment. Exposure to a blast wave produced with the 76.2 μm membrane significantly decreased the body temperature during the first hour in TBI-induced mice as compared to their sham controls (**Figure 4B**), indicative of a significant physiologic effect produced by TBI induction. Consistently, mice subjected to TBI using 76.2 μm membranes exhibited an acute, time dependent yet significant reduction in total body weight one-day post-TBI compared to sham (**Figure 4C**).

In order to examine the impact of TBI on behavioral outcomes, the effect of blast-induced TBI on acute locomotor activity was analyzed (**Figure 4D**). Adult, 3-month-old, male C57Bl/6J mice underwent TBI induction using 76.2 μm membrane or sham treatment and locomotor activity was monitored for 30 minutes three hours post-TBI. Exposure to a blast wave produced with the 76.2 μm membrane resulted in an acute, significant decrease in locomotor activity (**Figure 4D**).

Figure 1: Setup of murine blast wave model. (**A**-**C**) Representative images of the setup of the blast wave model for mice. Side view of the setup (**A**). Top view of the setup (**B**). 1, gas cylinder with a high flow gas regulator; 2, 9.53 mm high pressure hydraulic line and quick connect male and female attachments; 3, driver section of the shock tube; 4, driven section of the shock tube; 5, PVC pipe shield; 6, head placement area; 7, polyester membrane. The individual parts of the setup are installed on machine slide tables allowing for precise positioning of driver (3) and driven sections (4) in relation to subject undergoing injury induction. (**C**) Top view of setup with pressure sensor placements. Three sensors are located in one plane at the exit of the shock tube, 120 degrees apart (S1 - S3), to monitor the blast wave properties during the TBI induction. One sensor is installed at the head placement area (S4).

Figure 2: Schematic of murine overpressure shock tube. Precision-machined shock tube is made from high-tensile steel. Internal space of the driver section is angled at 6 degrees. Internal diameter of driver and driven section is 37 mm. Mating surfaces of driver-driven sections are precision-machined to ensure complete seal. The entire shock tube is industrially clamped to a machine slide table to ensure solid mounting and consistency of blast wave generation. At the exit of the driven section holes are drilled (in one plane, 120° apart) to install the three pressure sensors (indicated by *).

Figure 3: Pressure recordings from murine blast wave setup. (**A**,**B**) Peak pressure is scalable and dependent upon polyester membrane thickness. Pressure sensors were used to record peak pressures produced by the shock tube with helium gas and polyester membranes of 25.4, 50.8 or 76.2 μm thickness. (**A**) At the head placement area, the mean peak pressure produced with 25.4 μm membranes was 428 ± 15.9 kPa, with 50.8 μm membranes 637 ± 21.4 kPa and with 76.2 μm membranes 1257 ± 40.7 kPa (SEM, n = 7-12, one-way ANOVA followed by post-hoc Dunnett's comparison test, *** $P \leq 0.001$). (**B**) At the exit of the shock tube, the mean peak pressure recorded with 25.4 μm membranes was 164 ± 11.7 kPa, with 50.8 μm membranes 232 ± 11.7 kPa and with 76.2 μm membranes 412 ± 11.0 kPa (SEM, n = 7-12, one-way ANOVA

followed by post-hoc Dunnett's comparison test, ** $P \leq 0.01$, *** $P \leq 0.001$). (**C**) Representative graph of the pressure recording from the sensor at the head placement area (incident sensor) using a 76.2 μm membrane. The waveform is similar to that of a Friedlander wave, scaled in time/duration for murine subjects. (**D**) Representative graph of the pressure recording from 3 distinct sensors located at end of the driven section to determine the linearity/phase of waveform within the driven section. All three sensors (located 120 degrees apart) show a similar rise/fall duration indicating that the waveform leaving the driven section is similar in cross-section within the driven section. The blast wave was generated using a 76.2 μm membrane.

Figure 4: Acute in vivo effects of blast-induced TBI. (**A**) Moderate TBI, but not mild TBI increases righting reflex time (RRT). Adult, 3-month-old, male, wild-type C57Bl/6J mice were subjected to TBI procedures using the shock tube with helium gas and polyester membranes of 50.8 or 76.2 μm thickness or sham treatment. Immediately following injury or sham treatment, mice were allowed to recover and RRT was recorded. TBI induction with 50.8 μm membrane or sham treatment exhibited comparable levels of RRT. In contrast, TBI induction using a 76.2 μm membrane increases RRT, indicative of a loss of consciousness induced by the blast wave with the 76.2 μm membrane (SEM, n = 4-10, Sham RRT = 35.6 ± 2.0 s, 50.8 μm membrane RRT = 43.0 ± 4.3 s and 76.2 μm membrane RRT = 254.0 ± 40.2 s, one-way ANOVA followed by post-hoc Dunnett's comparison test, *** $P \leq 0.001$). (**B**) Moderate TBI significantly and transiently reduces core body temperature. Adult, 3-month-old, male, wild-type C57Bl/6J mice were subjected to TBI induction with 76.2 μm membranes or sham treatment. Their core body temperature was recorded for two hours. Baseline core body temperature was recorded prior to TBI induction. Blast-induced TBI with 76.2 μm membranes is associated with a significant drop in core body temperature within the first hour post-TBI. (SEM, n = 10, two-way repeated measures ANOVA, followed by post-hoc Bonferroni's multiple comparison tests, ** $P \leq 0.01$, *** $P \leq 0.001$). (**C**) Moderate TBI results in a transient reduction in body weight. Adult, 3-month-old, male C57Bl/6J mice were subjected to TBI procedures using 76.2 μm membranes or sham treatment. Subsequently, body weights were recorded for 5 days. Total body weight was significantly reduced one-day post-TBI (SEM, n = 7, two-way repeated measures ANOVA followed by post-hoc Bonferroni's multiple comparison tests, * $P \leq 0.05$). (**D**) Moderate TBI results in acute reductions in locomotor activity. Adult, 3-month-old, male C57Bl/6J mice were subjected to TBI procedures using 76.2 μm membranes or sham treatment. Three hours post-TBI locomotor activity was tracked for 30 minutes and quantified using video tracking software (SEM, n = 9-11, unpaired two-tailed t-test, ** $P = 0.01$).

DISCUSSION:

We present here a preclinical mild TBI model that is cost-effective, easy to set up and execute, and allows for high-throughput, reliable, and reproducible experimental outcomes. This model provides protective shielding to peripheral organs to allow for focused investigation into mild TBI mechanisms while limiting the confounding variables of systemic injury. In contrast, other blast models are known to inflict damage to peripheral organs[2,39,40]. Another advantage of this model is its capability to deliver the blast wave from any desired angle compared to the fixed position in other blast models[40]. This allows for focused anatomical studies to better

understand brain vulnerability.

In order to study human blast-related TBI, a relevant model for TBI should produce biomechanical forces comparable to those experienced by subjects during TBI induction. A clinically relevant model should also induce neurobiological, pathophysiological and behavioral outcomes observed in subjects suffering from mild TBI. In previous studies, the blast wave model presented here has been thoroughly examined[17,19,21], and numerous biophysical and neurobiological aspects reminiscent of human TBI, including blast wave dynamics and forces, neuroinflammation, axonal injury and microvascular damage have been evaluated. These studies have provided evidence that this preclinical blast wave model for TBI produces reliable and reproducible neurobiological and pathophysiological changes associated with clinical TBI.

Furthermore, with the increased incidence of mild blast TBI within the military population[7,8], this versatile rodent model for mild human TBI provides researchers with a valuable tool to investigate processes underlying blast-related TBI and explore novel therapeutic strategies. For example, our model demonstrates neurovascular complications, and highlights the importance for vascular intervention as a promising therapeutic approach[22,23,35]. Consistently, other preclinical models of blast TBI have also produced neurovascular effects associated with neurodegeneration and behavioral deficits[2,25,40-43].

Based on previous research[19,21-23], we have established that the blast wave model presented here may be well suited for the investigation into the pathophysiology and etiology of human concussion. Most preclinical TBI models do not permit head movement[44] even though the biomechanical properties associated with rapid head acceleration/deceleration are a predictive factor for the development of a concussion in humans[45,46]. Consistent with the model described herein, Goldstein and colleagues[14] showed that rapid head movement induced by blast forces are a prerequisite for the induction of behavioral deficits, possibly due to rotational forces and shearing. A better understanding of the pathophysiological changes that occur in mild TBI and in response to concussion would also help to determine clinical biomarkers and identify novel targets for the development of treatments for TBI.

Little is known about the pathophysiological changes and the disease progression following repetitive mild TBI (e.g., repetitive concussion experienced in sports). This preclinical model permits the study of repetitive mild TBI with little to no mortality. In contrast, some TBI models inflict severe injuries, and therefore it is often difficult, or inhumane, to induce further injury. In addition, severe injuries are often irreparable and the detection of subtle physiological changes may be precluded. This model also allows for the scalable investigation of various inter-injury intervals; a critical parameter for repetitive mild TBI that requires further characterization. After TBI, a CNS injury response is triggered that helps to protect brain integrity and prevent widespread neuronal cell death. The injury response may be, indeed, significantly impacted by the induction of another injury within a short time point after the initial injury. This model permits the investigation of the inter-injury interval, which is an important aspect of clinical trial design for repetitive mild TBI. Moreover, this scalable model allows for a rapid high-throughput workflow, which facilitates investigation of multiple parameters simultaneously, as well as the

evaluation of therapeutic activity of novel interventions.

One limitation of this model is the inability to control the properties of the blast wave between the tube exit and the animal's head. Although the blast wave is turbulent upon exit from the shock tube, the outcome measures are still reliable and reproducible with a consistent positioning of the rodent's head[18]. Therefore, it is important to keep the experimental settings (i.e., head position and distance from shock tube exit) constant between all studies. In order to optimize model design and protocol, waveform dynamics between the tube exit and the head placement area have been measured (**Figure 3**) and modeled using numerical simulations[18]. Future projects will integrate finite element modeling to determine how force dynamics transfer from the skull to meninges, to cerebrospinal fluid, and finally into the brain tissue. The complex interplay of force dynamics and biophysics and resulting physiological responses are important areas in TBI research that have been so far underexplored.

In summary, we present here a protocol and visualized experiment of a blast wave injury model that has been developed to investigate the effects of mild TBI. The collective experience of engineers, physicians, and biomedical scientists contributed to the optimization of its biophysical/physiological validity and neurobiological relevance. This model has been thoroughly validated and has already produced meaningful results, especially in understanding early dynamics of mild TBI[17,19-23]. Exploiting this preclinical model to further study mild TBI will significantly advance our understanding of the pathophysiology and etiology of TBI and contribute to the development of novel interventions for the benefit of patients suffering from TBI.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that they have no competing interests.

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Figure 1

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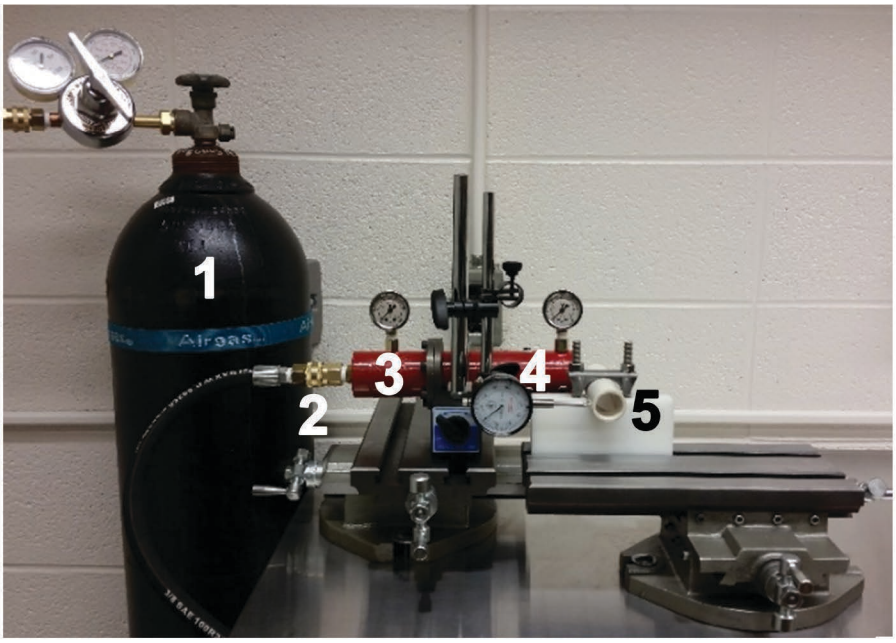
Fig 1

A



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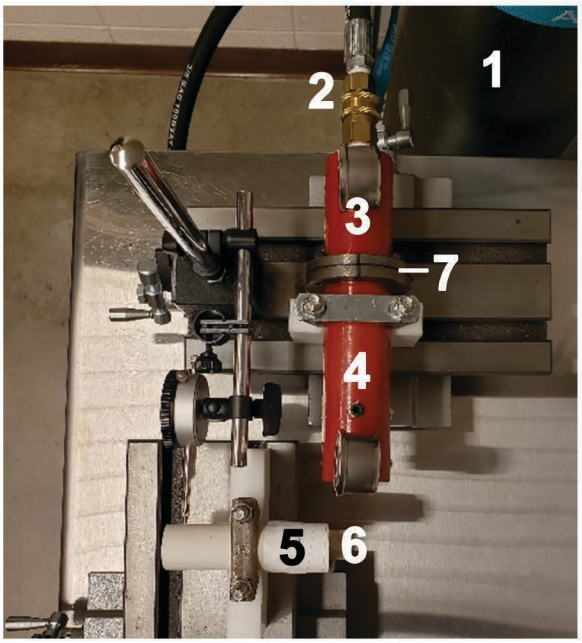

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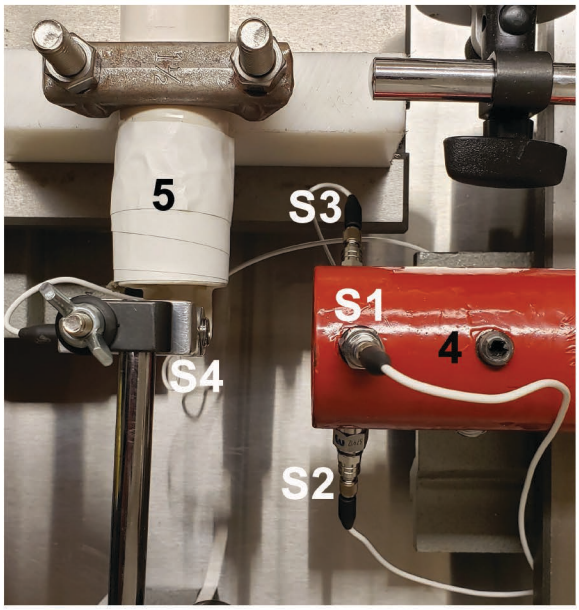

Figure 2

Fig 2

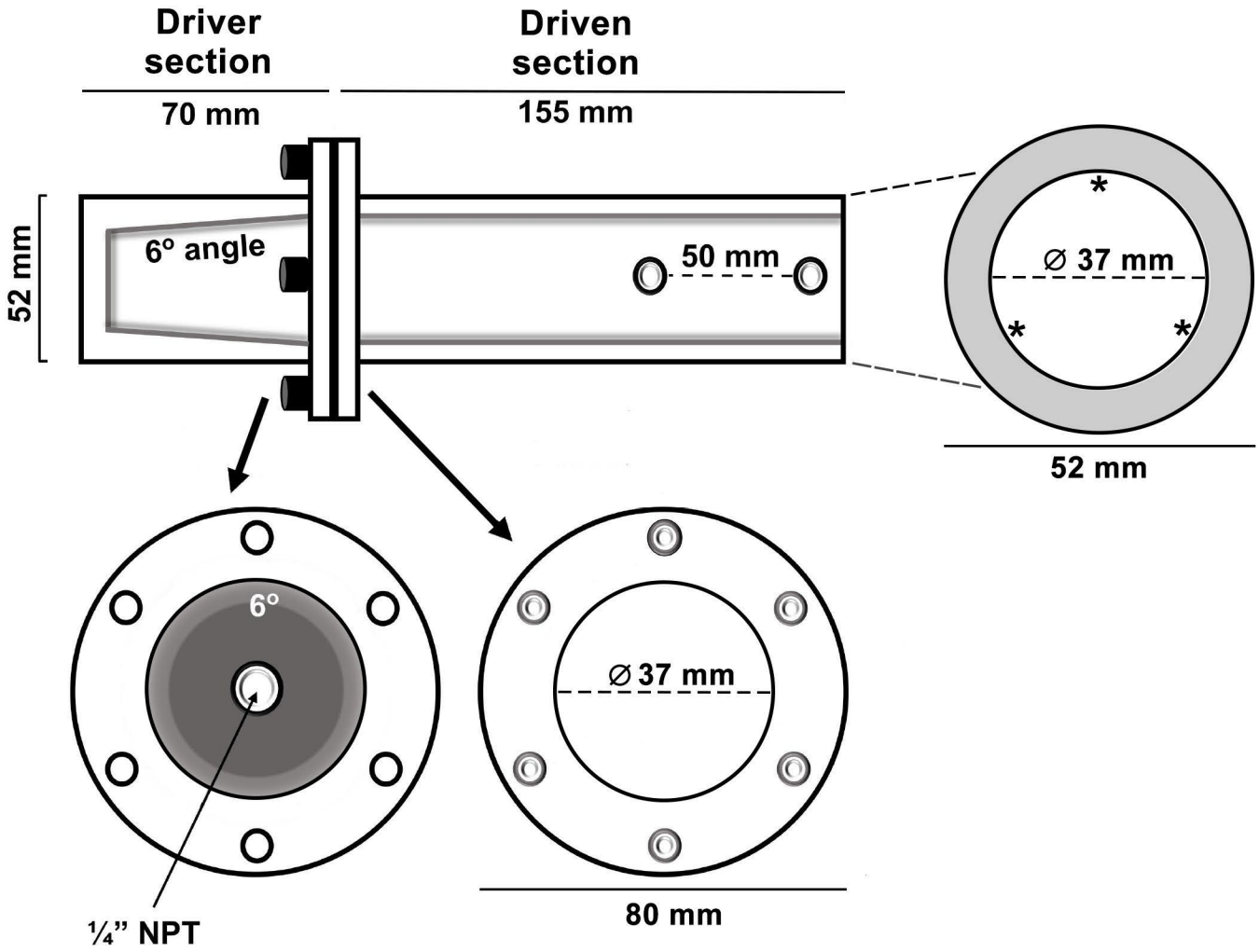

Figure 3

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Fig 2

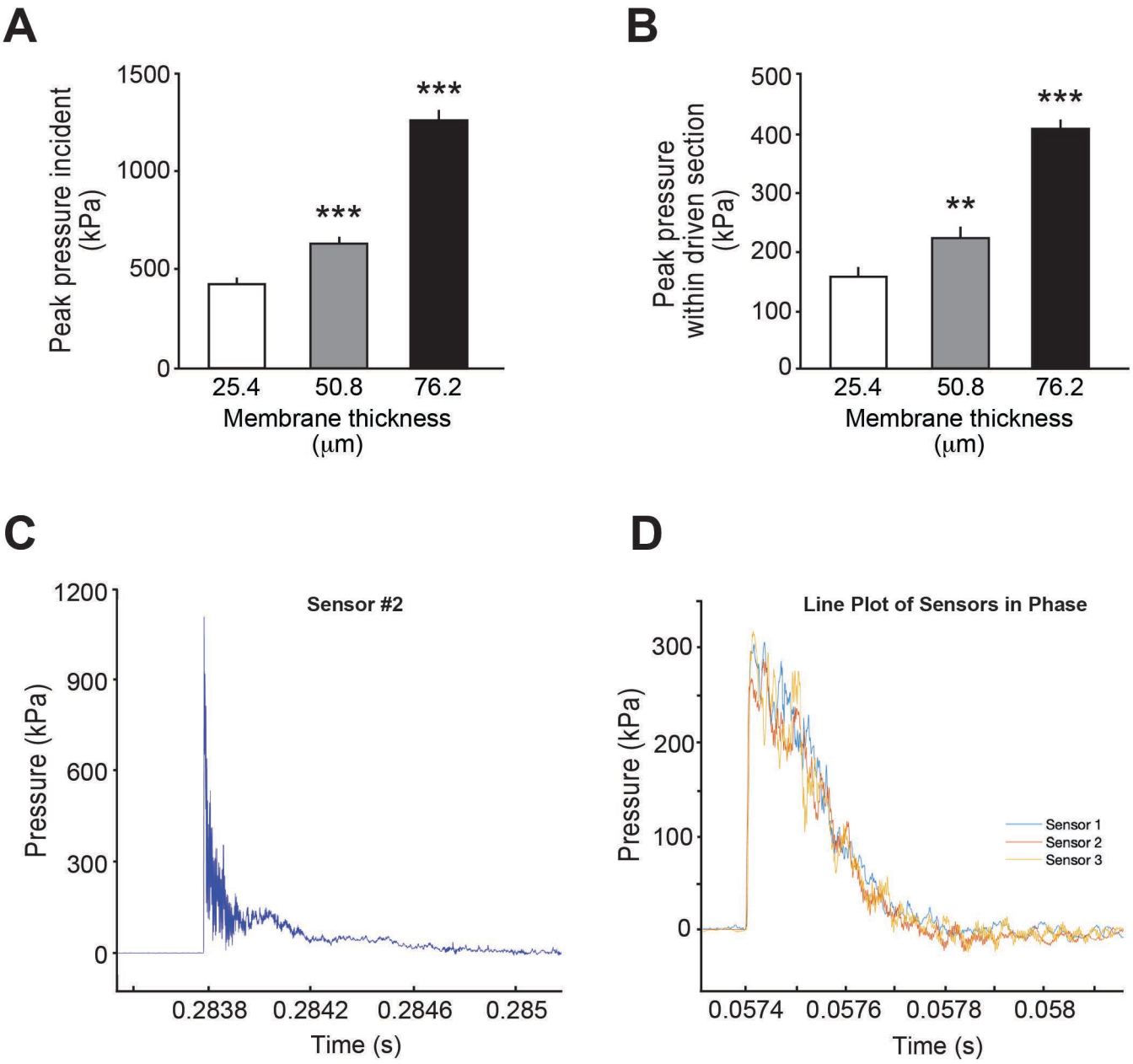

Figure 4

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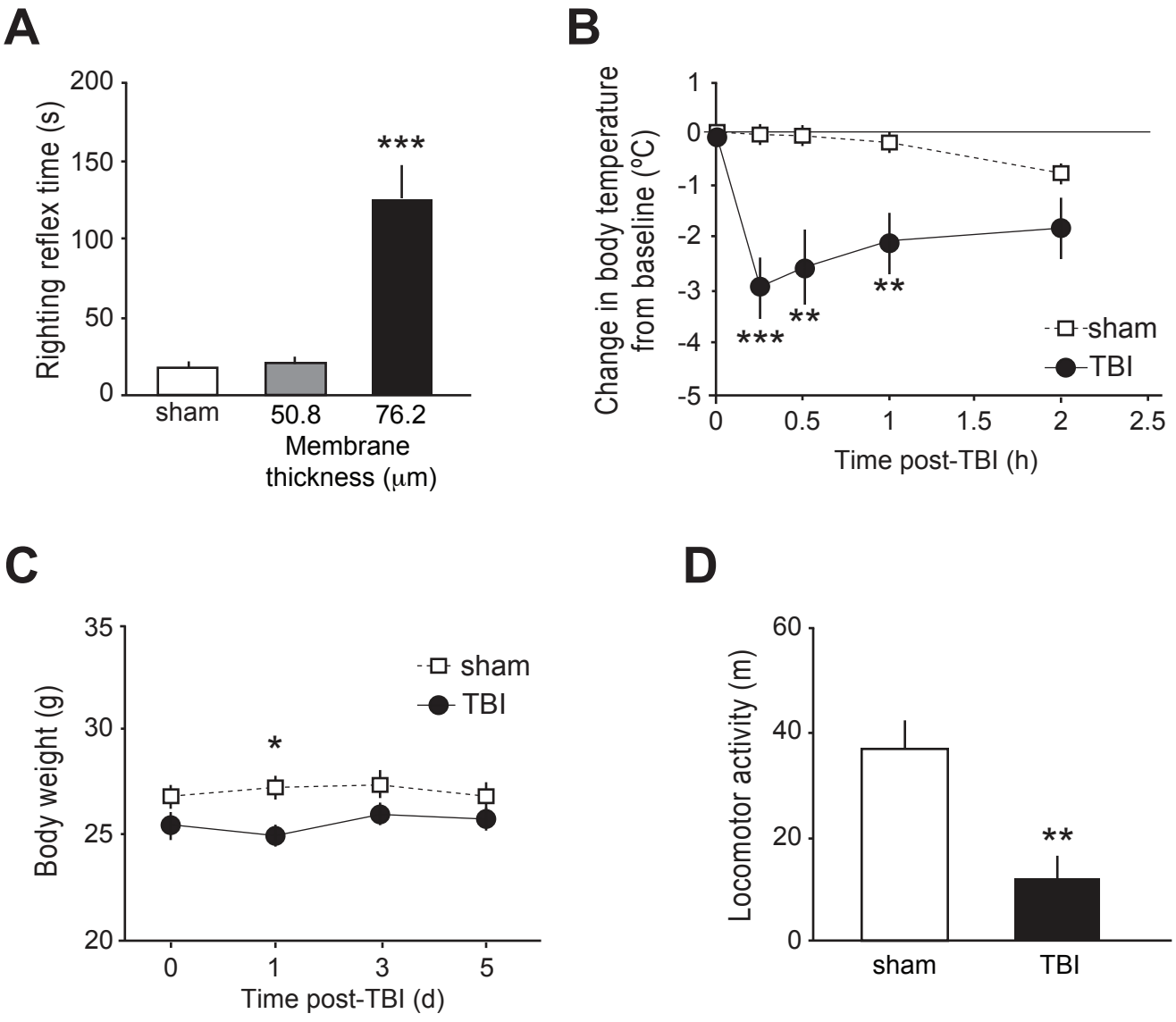
Fig 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3/8 SAE High Pressure Hydraulic Hose	Eaton Aeroquip	R2-6-6-36M	Available from Grainger
3/8" Quick Connect Female Plugs	Karcher	KAR 86410440	
3/8" Quick Connect Male Plugs	Karcher	KAR 86410440	
ANY-maze video tracking software	Stoelting Co.	ANY-maze software	
Clear Mylar membrane	ePlastics.com	POLYCLR0.003	http://www.eplastics.com/Plastic/Cl
Compound Slide Table (X2)	Grizzly Industrial	G5757	
Deadman Gas Control Ball Valve	Coneraco Inc.	71-502-01	"Apollo", Available from Grainger
Driver and driven section (murine)	own design/production	n/a	For further information please cont
Driver and driven section (rat)	own design/production	n/a	For further information please cont
Ear Muffs	3M	37274	Available from Grainger
Gas Regulator - Hi Flow 3500-600-580	Harris	3003539	
Helium Gas	AirGas	HE 300	Tanks are available in various size
Inhalation Anesthesia System	VetEquip	901806	
Input Module	National Instruments	NI 9223	
Isoflurane	Baxter	NDC 10019-360-40	Ordered by veterinarian
Laboratory Timer/Stopwatch	Fisher Scientific	50-550-352	
Labview version 12.0	National Instruments		Data Acquistion Software
Magnetic Dial Indicator/Micrometer	Grizzly Industrial	G9849	
MATLAB	MathWorks		Software for pressure recording an
Oxygen Regulator	Medline	HCS8725M	
PC for Data Processing	Dell		
Polyvinylchloride Tubing - 25.4 mm	FORMUFIT	P001FGP-WH-40x3	
Pressure sensors	PCB Piezotronics	102A05	
Receiver USB Chassis	National Instruments	DAQ-9171	
Sensor Signal Conditioner	PCB Piezotronics	482C series	
Stainless NSF-Rated Mounting Table	Gridmann	GR06-WT2448	
T Handle Allen Wrench - 3/16"	S&K	73310	

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REPLY TO REVIEWERS' COMMENTS

Manuscript: JoVE61244_R1

New Title: "Low-intensity blast wave model for preclinical assessment of closed-head mild traumatic brain injury in rodents"

Previous Title: "Compression wave injury model for preclinical assessment of closed-head mild traumatic brain injury in rodents"

We thank the Reviewers for their comments, suggestions and expert advise that were helpful in significantly improving the quality of the manuscript. The new version of the manuscript was thoroughly reworked to address the reviewers' comments and concerns. A detailed point-by-point discussion for each of the reviewers' comments is provided below.

Reviewer #1:

Manuscript Summary:
The authors are characterizing a new mild TBI closed head injury model using: Compression wave injury model.

Please accept the article

> We thank the reviewer for their review efforts and acceptance of the current version of the manuscript.

Reviewer #3:

Manuscript Summary:
The manuscript by Logsdon et al describes a rodent model of mild blast-induced traumatic brain injury using a novel bench-top blastwave generator. The device described in the protocol is relatively small and straightforward to use compared to the type of large shock-tube typically used to investigate blast TBI. These factors and the scalability allowing use in both rats and mice make the model of interest and potentially utilizable in many laboratories.

> We appreciate the reviewer's comments and positive evaluation of our novel blast wave model. As stated by the reviewer, it is also our hope that the model described herein will be of use for other groups, thereby contributing to the advancement of research into mild to moderate TBI.

Major Concerns:

The authors have addressed many of the concerns raised with reference to the first version. In particular details of the dimension of the device and additional experimental parameters have been provided that should be sufficient to reproduce the device.

We thank the reviewer for their positive evaluation of our revision and are glad to hear that the technical details are deemed sufficient to reproduce the device.

The manuscript is still missing quantitation or estimate of the head movement that is claimed to be a clinically relevant novel feature of this model, although it is stated that such characterization will be carried out. If this has been described in other publications the authors should make this clear.

We thank the reviewer for their keen observation that the specific quantitation of the head movement is not included within the current manuscript. We have now amended the manuscript and deemphasized the importance of the head movement in the 'Abstract', 'Introduction' and Protocol' section.

We agree that these measurements are important for the current model, so much so that we are currently working with a group that specializes in aerospace engineering (University of Cincinnati) to characterize both the angular head motion and forces exerted upon the rodent's head throughout the exposure period to the blast wave. These analyses will utilize strain gauges and sensors typically utilized in the area of jet propulsion (with ignition-type shock tubes) to thoroughly characterize the actual kinetics, forces and angular momentum imparted upon murine subjects. We believe these analyses are very important due to the published and known effect of head movement being necessary for behavioral effects elicited by blast forces in rodents (Goldstein *et al.* 2012. Science Translational Medicine; see also 'Discussion'). We have cited this work and included a section on the importance of the head movement in the 'Discussion'.

At the current time, we do have unpublished observations/data of reproducible head motion from analyses of a ball and spring model using our blast wave setup (that we can provide, if requested). However, these observations/data do not truly recapitulate the forces imparted on a rodent skull. In our ongoing research, we are also utilizing high speed imaging (Schlieren imaging) of blast waves to correlate the kinetics of the blast waves themselves to the actual head rotation/motion in collaboration with our engineering colleagues mentioned above. These data will be available in a future collaborative manuscript but are beyond the scope of the current purely methods oriented manuscript.

Throughout the manuscript there is frequent mention of compression wave injury and the relevance of this to mild TBI in general. In reality 'compression waves' of the Friedlander waveform shown are really low intensity blast waves. The model is therefore a model of mild blast TBI (including the Friedlander waveform) - with some features relevant to other types of TBI (eg. coup/contrecoup) due to free head movement.

The text still needs to be modified to reflect this.

We agree with the reviewer and would like to thank for their clarification and straightforward statement of this crucial point. There had been, and still is, much contention in the blast field over the relevance of many rodent models for blast-induced TBI due to the nature of the blast waveforms that actually impact the rodent. As noted by the reviewer, our model does produce the classic Friedlander waveform, which is a low-intensity blast wave. To reflect this comment, we have modified the text within the entire manuscript accordingly.

Reviewer #4:

Manuscript Summary:
The present research includes a detailed protocol to develop a rodent model of blast exposure using compressed air. Paper is well written, detailed but the introduction is totally irrelevant to the type of TBI. Novel data concerning the effects of blast exposure on body temperature. This reviewer was not aware of this.

We thank the reviewer for their positive evaluation of the blast model, the manuscript and the novel data on body temperature. We agree that the 'Introduction' did not appropriately cover the topic and we have revised the entire text accordingly.

Major Concerns:
The introductions should be targeted to military TBI as the described procedure in reality is totally related to blast-exposure. There is a universe difference between mechanical TBI and blast-induced TBI. The work is very nice and deserves to be published, but it should be complemented with an adequate introduction and discussion related to blast exposures.

We agree with the reviewer that this model is relevant for military TBI exposure. We have modified the introduction to reflect the connection of this blast wave model to blast-exposure and military TBI. We have included text stating the relationship between blast exposure and CNS dysfunction, the increased incidence of blast-elicited TBI in US servicemembers and the importance of preclinical TBI models for the development of novel, effective TBI therapeutics. We have added a section to the 'Discussion' to cover blast exposure.

Minor Concerns:
Please include pressures in kPa and include the wide literature describing the neurovascular degenerative processes associated with blast exposure.

We have amended the graphs and text within the manuscript to include kPa values for proper comparison to other blast-related publications. We have also included multiple references within the discussion that describe the contributions of blast injury to neurovascular degeneration and how this model, and similar models, can be used to examine this effect.

Reviewer #5:

Manuscript Summary:
This paper describes an attempt to use something closer to a laboratory model for blast injury as a preclinical model for mild TBI caused by blunt force trauma or other accelerations.

Major Concerns:
The abstract and intro misrepresent the utility of the controlled cortical impact and lateral fluid percussion models in the study of mild TBI - the shortcomings of these models are exaggerated and the breadth and scope of their applicability are minimized. This seems intended to make the proposed new method more urgently needed or important than it has been shown to be.

We agree with the reviewer that CCI and LFPI are well-established TBI models that are useful for the investigations of human TBI. We have amended the 'Abstract' and 'Introduction' accordingly. We did not intend in any way to trivialize the contributions of both CCI and LFPI to the TBI field.

The statement from lines 563-564 in the discussion, "compression waves are the most clinically relevant form of mild TBI to look at acceleration/deceleration" is not supported. Neither the present study nor the citations provide validation for the method as clinically relevant for mild TBI from acceleration and deceleration, much less their superiority to existing and well-validated experimental models.

We agree with the reviewer's comments and have amended the text in the entire manuscript accordingly. The statement from lines 563-564 has been removed from the 'Discussion' as there exists no validation for this claim. Instead, we emphasize that the blast wave model presented here is a preclinical model with utility to investigating neurobiology, pathophysiology and/or behavior that is reminiscent of clinical problems encountered in blast-induced TBI.

The method is new, and it may eventually prove useful in some contexts - but I expect the usefulness will be in its ease of use and repeatability rather than in being the closest representation to acceleration induced mild TBI. Direct penetration of the blast wave through the skull and into the brain may always be a confounding factor.

We thank the reviewer for their positive assessment of the method and its potential value for future research. We agree with the reviewer that the blast wave penetration through the skull may cause direct injury, thereby representing a confounding factor. We have now included a discussion of this confound and how the effects of direct blast wave penetration vs. head rotation/acceleration for the development of neurological deficits can be evaluated by using head fixation in this blast wave model.

As is true for most human conditions, rodent models often replicate only certain aspects of the clinical manifestations. Thus, the study of a combination of TBI models will ultimately be required to critically advance our understanding of the human condition.

Minor Concerns:
Lines 173-174 suggest there are rigid, mutually exclusive definitions of blast waves, compression waves, and shock waves; whereas, this does not appear to be the case. Very similar pressure transients appear to be given different names based more on the context in which they are discussed rather than rigorous and distinct criteria by which they are defined. The "compression waves" of the present study may well be referred to as "blast waves" in a study purporting to be more relevant to blast induced TBI.

We thank the reviewer for their comments regarding waveforms and their notion that the compression waves in this study could be referred to as blast waves. We absolutely agree with this comment and have modified the text throughout the entire manuscript accordingly. We also included new sections that underscore the relevance of this blast wave model to blast-induced TBI.