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Repeated Primary Blast Injury Causes Delayed Recovery, but not Additive Disruption, in an *In Vitro* Blood–Brain Barrier Model

Christopher D. Hue,[1] Siqi Cao,[1] Cameron R. "Dale" Bass,[2] David F. Meaney,[3] and Barclay Morrison III[1]

Abstract

Recent studies have demonstrated increased susceptibility to breakdown of the cerebral vasculature associated with repetitive traumatic brain injury. We hypothesized that exposure to two consecutive blast injuries would result in exacerbated damage to an *in vitro* model of the blood–brain barrier (BBB) compared with exposure to a single blast of the same severity. Contrary to our hypothesis, however, repeated mild or moderate primary blast delivered with a 24 or 72 h interval between injuries did not significantly exacerbate reductions in transendothelial electrical resistance (TEER) across a brain endothelial monolayer compared with sister cultures receiving a single exposure of the same intensity. Permeability of the barrier to a range of different-sized solutes remained unaltered after single and repeated blast, supporting that the effects of repeated blast on BBB integrity were not additive. Single blast exposure significantly reduced immunostaining of ZO-1 and claudin-5 tight junction proteins, but subsequent exposure did not cause additional damage to tight junctions. Although repeated blast did not further reduce TEER, the second exposure delayed TEER recovery in BBB cultures. Similarly, recovery of hydraulic conductivity through the BBB was delayed by a second exposure. Extending the interinjury interval to 72 h, the effects of multiple injuries on the BBB were found to be independent given sufficient recovery time between consecutive exposures. Careful investigation of the effects of repeated blast on the BBB will help identify injury levels and a temporal window of vulnerability associated with BBB dysfunction, ultimately leading to improved strategies for protecting warfighters against repeated blast-induced disruption of the cerebral vasculature.

Key words: blood–brain barrier; endothelial cells; repeated blast injury; shock tube; traumatic brain injury

Introduction

Prevalent use of improvised explosive devices (IEDs) in recent military conflicts has motivated study of the underlying neuropathological mechanisms associated with mild traumatic brain injury (mTBI) resulting from one or more exposures to blast overpressure.[1–3] Epidemiological studies have reported that repeated exposure to mild blast overpressure may potentially increase the burden of neurobehavioral and cognitive deficits, in part, by lowering the threshold for damage and establishing a temporal window of heightened vulnerability.[4–6] In the military setting, personnel exposed to low-intensity blasts often exhibit mild symptoms and may return to duty without sufficient recovery time, placing them at greater risk of sustaining additional blast injuries that may worsen ongoing pathobiological cascades.[5,7,8] Although potential effects of multiple blast exposures pose a major challenge to the military health care system, results of clinical and experimental investigations have not conclusively demonstrated exacerbated neuropathological, cognitive, or inflammatory outcomes after repeated blast exposure.[1,4,7–14]

Studies using impact- and inertia-driven models of repetitive brain injury (non-blast) have shown that repeated insults delivered within specific time frames can aggravate brain pathology; however, only a limited number of studies have addressed the potential for concomitant blood–brain barrier (BBB) dysfunction.[15] Experimental and human traumatic brain injury (TBI) case studies have shown that repeated exposure to mTBI can lead to dramatic cumulative deficits in behavior, cognition, and cerebral metabolism when they occur within hours to days after the initial insult.[15–20] Athletes receiving a second injury while still symptomatic from a previous head injury presented with evidence of cerebral vascular engorgement, consistent with the clinical scenario of second-impact syndrome whereby exposure to a single mTBI results in elevated risk for severe damage induced by subsequent mTBIs.[5,21]

[1]Department of Biomedical Engineering, Columbia University, New York, New York.
[2]Department of Biomedical Engineering, Duke University, Durham, North Carolina.
[3]Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania.

Although previous work from our laboratory and others has assessed the acute effects of a single primary blast on the BBB,[7,22–26] only recently have studies started to shed light on the pathological complexity associated with repeated blast-induced TBI (bTBI) on the cerebral microvasculature. In a recent study of officers from the Swedish Armed Forces, repeated exposure to the firing of heavy weapons and explosives produced no neurochemical evidence of neuronal or BBB damage.[9] In contrast, primary blast injury in rats induced nitrosative and oxidative damage in the BBB after repeated exposure to low-intensity shockwaves, as well as reduced the tight-junction proteins zonula occludens-1 (ZO-1), claudin-5, and occludin.[7] Others have reported that the pathological changes observed in rats exposed to multiple mild blast injuries were not cumulative, despite findings of significant neuronal, glial, and vascular damage after a single mild exposure.[5] Therefore, additional work is needed to develop a more detailed understanding of the cerebrovascular changes that arise after multiple blast injuries and their relation to the neuropathological and behavioral changes associated with bTBI.[9,13,15]

In this study, we investigate the consequences of repeated bTBI by subjecting an *in vitro* model of the BBB (consisting of a monolayer of brain endothelial cells)[24] to controlled primary blast-loading at realistic exposure levels.[22,24,27] We test the hypothesis that exposure to two blast injuries administered within a short time frame leads to cumulative functional deficits in the BBB compared with a single exposure. The resulting outcome on BBB integrity was found to be dependent on the severity of the injury and time interval between insults. We report that damage to the BBB after two blast injuries, as opposed to one, was not additive. Importantly, we find that repeated blast delayed the spontaneous recovery of BBB function. With a prolonged interinjury interval, the effects of multiple injuries on the BBB were found to be independent given sufficient recovery time between consecutive injuries. Defining the window of vulnerability for damage to the cerebral microvasculature may hold important implications for mandatory resting periods for service members exposed to blast, before returning to duty.

Methods

BBB cell culture model

Monolayers of immortalized mouse brain endothelial cells, bEnd.3 (ATCC, Manassas, VA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% newborn calf serum (Thermo Scientific, Logan, UT) and 4 mM GlutaMAX (Life Technologies, Carlsbad, CA). A total of 60,000 cells were seeded in Transwell inserts (1.12 cm^2 surface area) pre-coated with poly-L-lysine, and grown to confluence to represent *in vitro* models of the BBB.[24] Cell cultures were maintained for 7 days before experimentation to achieve confluent, integral monolayers in a cell-culture incubator at 37°C and 5% CO_2/95% O_2, and were fed with new medium every 2–3 days.[22,24]

Repeated exposure of BBB to primary blast injury

As described previously in detail, an *in vitro* bTBI model composed of a shock tube and fluid-filled sample receiver was used to expose BBB cultures to controlled primary blast injury.[22,24] For repeated blast exposures, *in vitro* BBB cultures were subjected to two consecutive blasts at pre-determined severity levels, separated by either a 24 h or extended 72 h interval. Before injury, cultures were placed in sterile bags (Whirl Pak, Fort Atkinson, WI) filled with medium pre-warmed to 37°C. In accordance with previously published methods,[22,24] samples were then loaded into the fluid-filled receiver and secured above a perforated polytetrafluoroethylene (PTFE) membrane held at a pre-determined depth. Cultures were oriented in the receiver to allow propagation of the fluid pressure transient in the direction perpendicular to the endothelial monolayer. The double injury group was exposed to an initial blast (injury 1) as well as a subsequent blast (injury 2). The single injury group was exposed to an initial blast (injury 1), but treated as a sham control for the subsequent blast (injury 2). Sham controls were processed identically to blast-injured cultures at both injury 1 and injury 2, but were not exposed to blast overpressure at either injury time point.

Primary blast overpressure was generated using a 76 mm-diameter shock tube with an adjustable-length driver section (25 mm used for current study) pressurized with compressed helium gas, and a 1240 mm-long driven section. A detailed schematic of the blast-injury device, along with example pressure traces recorded in the open-tube configuration and in the fluid-filled receiver, are described in a previous investigation.[24] The incident pressure of the shockwave was recorded by pressure transducers (8530B, Endevco, San Juan Capistrano, CA) flush-mounted at the exit of the tube. The fluid pressure history located at the level of the BBB culture was recorded by a pressure transducer (8530B, Endevco) flush-mounted to the interior of the receiver test column.

The blast severities ranged from mild to moderate intensity levels (Table 1), previously reported to cause acute *in vitro* BBB disruption.[24] The mild exposure was associated with nominal acute deficits in transendothelial electrical resistance (TEER), while the moderate exposure was associated with greater decreases in TEER signifying moderate acute damage to the barrier.[24] The biomechanical injury parameters were characterized by peak incident overpressure, duration, and impulse determined in the sample receiver and in the open-tube configuration. Because open-tube pressure recordings represent independent measurements uninfluenced by interactions with structures positioned downstream, all blast exposure conditions tested in this study will be identified by their open-tube parameters, unless otherwise noted.

Measurement of transendothelial electrical resistance (TEER)

The functional integrity of the BBB can be determined by TEER, a sensitive measure of ion flux through the brain endothelial monolayer.[24,28,29] All TEER values were recorded by placing the *in vitro* BBB model into an Endohm-12 electrode chamber connected to an EVOMX Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL). TEER was measured immediately before delivery of the first blast, and approximately 5 min after exposure to the first and second blasts (or sham exposures). For measuring time course changes, TEER was recorded 5 min after the

Table 1. Experimental Repeated Blast Injury Parameters

	Peak overpressure (kPa)	*Duration (ms)*	*Impulse (kPa*ms)*	*Severity*
Open-tube (in air)	377 ± 8	0.89 ± 0.01	96 ± 1.5	Mild
Receiver (in fluid)	902 ± 12	1.38 ± 0.02	483 ± 2.6	
Open-tube (in air)	402 ± 9	0.92 ± 0.01	118 ± 2.2	Moderate
Receiver (in fluid)	1196 ± 26	1.35 ± 0.02	580 ± 6.6	

Primary blast-loading conditions for repeated blast exposure were characterized by peak incident overpressure, duration, and impulse. Parameters were measured in the open-tube configuration and in the fluid-filled sample receiver in close proximity to the sample (mean ± standard error of the mean; $n \geq 3$).

first (day 1) and second (day 2) blast injuries, and once every 24 h until day 6 from the initial injury. For experiments involving an extended interinjury interval, TEER was recorded 5 min after the first (day 1) blast injury, and once every 24 h thereafter, including 5 min after the second (day 4) blast injury.

As previously described, each individual TEER measurement was corrected for the TEER of a cell-free Transwell insert and adjusted to account for the membrane surface area.[22,24] All TEER data presented in this article are normalized as the ratio of each culture's post-injury TEER values to its individual TEER value before the initial insult. For time course measurements, the TEER values of injured cultures were compared with age-matched and time point-matched sham controls. In accordance with our previous work,[22,24] cultures exhibiting TEER values less than 15 Ω*cm^2 before experimentation were deemed to be in suboptimal health and excluded from the study.

Measurement of solute permeability

The intact BBB forms a restrictive barrier to the paracellular permeation of larger solutes.[30] Quantifying permeability of a range of molecular weights across the endothelial monolayer provides one method to assess BBB dysfunction after repeated blast injury. Approximately 30 min after exposure to the second blast injury or sham exposure, fluorescein-labeled dextrans of 3 and 10 kDa (Life Technologies; ex/em: 494/521 nm), and Texas Red®-labeled dextrans of 40 kDa (Life Technologies; ex/em: 595/615 nm) were added to the upper compartment above the endothelial monolayer. Every 60 min for 4 h, 100 μL of medium from the compartment below the endothelial culture was collected and replaced with 100 μL of fresh medium. The change in volume associated with sampling at multiple time points was accounted for when calculating the change in solute concentration over time. Fluorescence of the collected samples was quantified using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA). As previously described, the diffusive solute permeability (P, cm/s) was calculated using equation 1.[24,28,29]

$$P = \frac{\frac{\Delta C_B}{\Delta t} \times V_B}{S \times C_T} \quad (1)$$

where, $\frac{\Delta C_B}{\Delta t}$ represents the change in concentration of dextrans over time in the compartment below the endothelial culture, V_B the volume contained in the compartment below the endothelial culture, S the surface area of the culture, and C_T is the concentration in the compartment above the culture.

Assessment of tight junction morphology

The presence and morphology of tight junction proteins, ZO-1 and claudin-5, between adjacent endothelial cells serve as indicators of BBB integrity.[7,24,28,29,31] Approximately 30 min after exposure to the second blast injury or sham exposure, the BBB endothelial cultures were fixed and incubated with either anti-ZO-1 rabbit polyclonal antibody or anti-claudin-5 mouse monoclonal antibody (Life Technologies). In separate experiments to determine the presence of each tight junction protein, ZO-1 was detected using the Alexa Fluor 488 anti-rabbit secondary antibody and claudin-5 was detected using the Alexa Fluor 488 anti-mouse secondary antibody (Life Technologies). Endothelial monolayers were also incubated with 4′, 6-diamidino-2-phenylindole (DAPI; Life Technologies) to detect individual cell nuclei and to quantify the number of cells in each BBB culture. All antibodies used were manufactured from the same lot and applied at the same post-injury time point.

Following the immunostaining procedure, cultures were imaged using an Olympus IX81 (Olympus America, Center Valley, PA) fluorescence microscope and MetaMorph software (Molecular Devices, Sunnyvale, CA). As previously described,[24] the degree of tight junction immunostaining was measured by applying the same threshold for ZO-1 or claudin-5 immunofluorescence to each of five randomly selected images taken from every sham control or injured culture. Immunostaining for ZO-1 or claudin-5 was quantified as the area-percentage of an image exhibiting fluorescence above an identical threshold, normalized to the total number of endothelial cells in each image.

Measurement of hydraulic conductivity

Exposure to primary blast has been found to enhance the leakiness of the BBB, which promotes vascular fluid influx and brain edema.[7] Hydraulic conductivity provides a quantitative measure of water flux through the BBB model.[24,28,29] Approximately 15 to 30 min after exposure to the second blast injury or sham exposure, cultures were placed in a custom-built permeability device similar to one described previously.[24,28,29,32] Each Transwell culture was tightly secured in a polycarbonate chamber connected to a lower fluid reservoir, establishing a known hydrostatic pressure (approximately 20 cm H_2O) across the endothelial monolayer. Hydrostatically induced fluid flow was quantified by tracking the linear displacement of an air bubble through a calibrated glass tube. Hydraulic conductivity (L_p, cm/s/cmH_2O) was calculated using equation 2.[24,28,29]

$$L_p = \frac{\frac{\Delta x}{\Delta t} \times F}{S \times \Delta P} \quad (2)$$

where, $\frac{\Delta x}{\Delta t}$ represents the linear displacement of the bubble over time, F the fluid volume of the calibrated glass tube, S the surface area of the culture, and ΔP the hydrostatic pressure across the endothelial culture.

Statistical analysis

Repeated-measures statistical analysis followed by one-way analysis of variance (ANOVA) and Bonferroni *post hoc* tests to sham controls was used to determine the overall effect of blast on TEER response for repeated exposures delivered 24 or 72 h apart, as well as for temporal TEER recovery associated with repeated blast exposures. Hydraulic conductivity and tight junction immunostaining data were analyzed statistically by one-way ANOVA followed by Bonferroni *post hoc* tests to sham controls. Solute permeability data were analyzed by independent samples t tests between sham and injured cultures. (SPSS v. 20, IBM, Armonk, NY, significance $*p < 0.05$)

Results

Severity-dependent TEER response after repeated blast

The *in vitro* BBB model was subjected to two blasts delivered 24 h apart at a severity level previously determined to result in mild functional disruption of the endothelial monolayer (377 kPa peak overpressure, 0.89 ms duration, and 96 kPa*ms impulse).[24] In the single injury group receiving one mild blast (injury 1), TEER decreased significantly compared with sham to 91 ± 3% (mean ± standard error of the mean [SEM]), and was not significantly different at 92 ± 6% after the second sham injury time point (Fig. 1A). In the double injury group receiving two mild blasts delivered 24 h apart, TEER was significantly decreased compared with sham to 84 ± 4% after injury 1 and 78 ± 4% after injury 2 (Fig. 1A). There was no significant difference in TEER between the double and single injury groups after the second injury time point, indicating mild effects of repeated exposure to a mild intensity blast. Sham control TEER levels were statistically unchanged at 102 ± 2% after the first sham exposure and 99 ± 6% after the second (Fig. 1A).

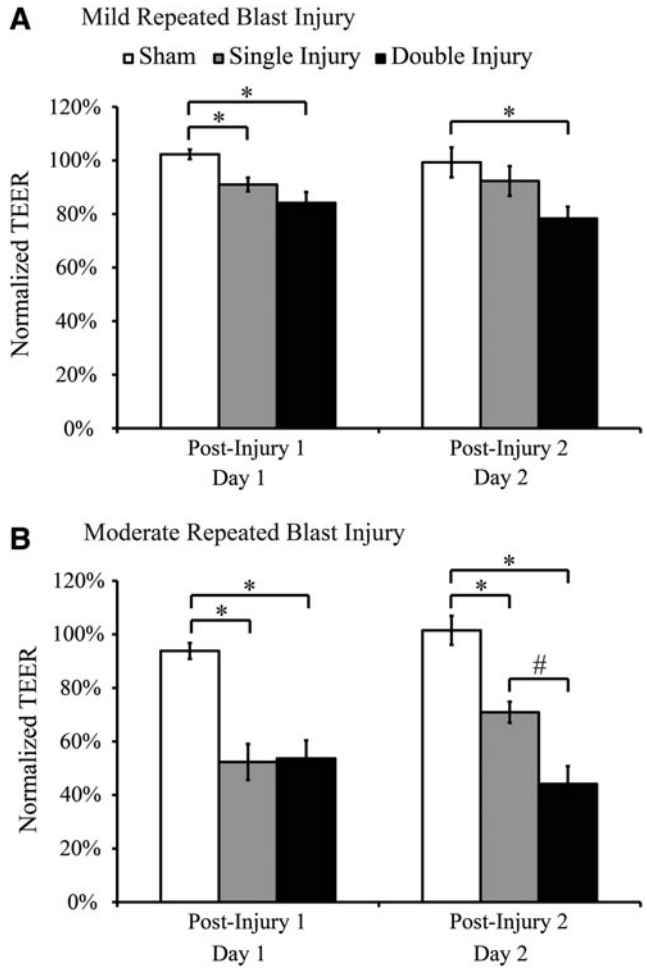

FIG. 1. Acute transendothelial electrical resistance (TEER) response of *in vitro* blood–brain barrier (BBB) model to repeated mild and moderate blast injuries. (**A**) Mild, sustained disruption of the BBB after repeated mild blast with a 377 kPa peak overpressure, 0.89 ms duration, and 96 kPa*ms impulse. (**B**) Pronounced, sustained disruption of BBB after exposure to repeated moderate blast with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse. ($^{*,\#}p < 0.05$; ± standard error of the mean; Sham $n = 12$; Single Injury $n \geq 11$; Double Injury $n = 12$).

When the blast severity was increased to a level previously determined to result in moderate functional disruption of the endothelial monolayer (402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse),[24] more dramatic compromises in BBB integrity were observed. In the single injury group receiving one moderate blast, TEER decreased significantly compared with sham to 52 ± 7% after the initial injury and remained significantly depressed at 71 ± 4% after the second sham injury; compared with after the first injury, TEER was slightly increased (not significantly) after the second sham injury because of spontaneous recovery of the monolayer (Fig. 1B). In the double injury group (24 h interinjury interval), TEER was significantly decreased to 54 ± 7% after injury 1 and similarly to 44 ± 7% after injury 2 (Fig. 1B), demonstrating sustained, but not additive, disruption of the monolayer with repeated injuries. TEER of the double injury group was significantly more depressed than the single injury group after the second injury time point because of persistent damage in the double injury group and partial recovery in the single injury group. TEER of sham controls was consistently high at 94 ± 3% and 101 ± 5% after the first and second sham injuries, respectively (Fig. 1B). It is important to note that previous characterization of our blast injury model confirmed the absence of any significant cell death or cell detachment from the Transwell membrane after blast exposure at higher severity levels than those tested in the current study.[22,24]

Unaltered solute permeability after repeated blast

Despite slightly elevated permeability of the 3 kDa dextran in the single and double injury groups after exposure to moderate blast compared with sham, there was no significant difference among the experimental groups for the 3, 10, or 40 kDa molecular weight tracers (Fig. 2). Solute permeability of all groups was measured 30 min after exposure to the second blast injury or sham exposure. As a positive control, endothelial monolayers were exposed to a single blast with a 571 kPa peak overpressure, 1.06 ms duration, and 186 kPa*ms, previously reported to significantly increase permeability of 10 kDa dextrans through the barrier.[24] After acute exposure to the same blast level in the current study, solute permeability significantly increased to $1.77 \pm 0.25 \times 10^{-6}$ cm/s compared with $0.90 \pm 0.05 \times 10^{-6}$ cm/s in sham controls (data not shown), which is in agreement with our previously published data.[24]

Tight junction disruption after repeated blast

Widespread and integral expression of tight junction proteins was indicated by high levels of ZO-1 and claudin-5 staining in sham control cultures (Fig. 3A, E). As described previously, visual inspection of bright-field and immunostained images of sham control cultures confirmed the formation of confluent monolayers exhibiting spindle-shape morphology that was characteristic of bEnd.3 cells and the brain endothelial cell phenotype.[22,24,28,29,31,33] Approximately 30 min after exposure to the second moderate injury or sham exposure, tight junction protein-staining for the single and double injury groups appeared less intense and slightly more punctate in morphology compared with sham controls.

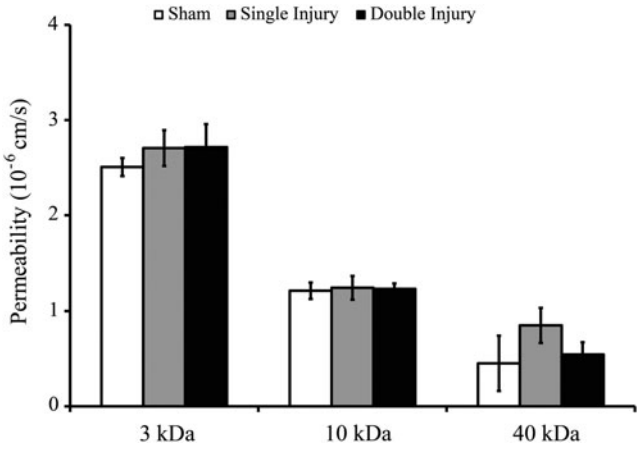

FIG. 2. Unaltered solute permeability after repeated blast injury in blood–brain barrier cultures. After exposure to moderate levels of blast with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse, no significant difference in solute permeability was observed between sham and the single or double injury groups. ($^{*}p < 0.05$; ± standard error of the mean; Sham $n = 10$; Single Injury $n = 10$; Double Injury $n \geq 9$).

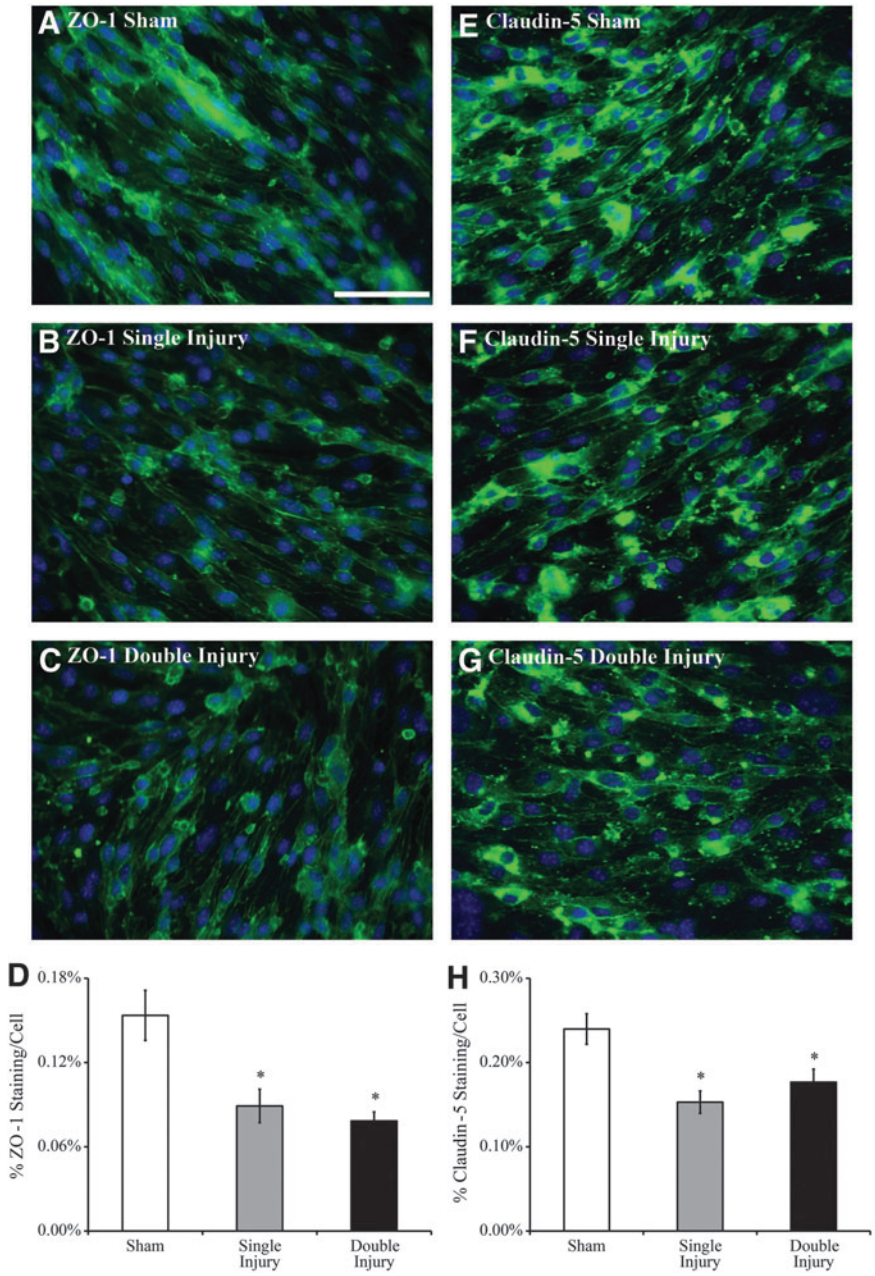

FIG. 3. Immunostaining of tight junction proteins ZO-1 (green) and claudin-5 (green) after repeated moderate blast injury with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse. (**A, E**) ZO-1 and claudin-5 staining was high in sham cultures, together indicating the presence of well-formed tight junctions. (**B, F**) Exposure to a single blast injury compromised ZO-1 and claudin-5 staining compared with sham controls. (**C, G**) Exposure to repeated blast injury compromised ZO-1 and claudin-5 staining compared with sham controls, but not compared with the single injury group. (**D, H**) Quantified ZO-1 and claudin-5 staining in the single and double injury groups was significantly reduced compared with sham, consistent with qualitative depictions in immunofluorescence images. (*$p < 0.05$; ± standard error of the mean; Sham $n = 6$ cultures (30 images); Single Injury $n = 6$ cultures (30 images); Double Injury $n = 6$ cultures (30 images) for ZO-1 or claudin-5; Scale bar = 70 μm). Color image is available online at www.liebertpub.com/neu

The area-percentage of ZO-1 immunostaining per cell was significantly decreased to 0.09 ± 0.01% in the single injury group and to 0.08 ± 0.01% in the double injury group, compared with 0.15 ± 0.02% in sham controls (Fig. 3D). Levels of ZO-1 immunostaining were not significantly different between the single and double injury groups, showing no cumulative disruption of the ZO-1 tight junction protein. Similarly, the area-percentage of claudin-5 immunostaining per cell was significantly decreased to 0.15 ± 0.01% in the single injury group and to 0.18 ± 0.02% in the double injury group, compared with 0.24 ± 0.02% in sham controls (Fig. 3H). Levels of claudin-5 immunostaining were not significantly different between the single and double injury groups. Overall, the altered tight junction morphology in both single and double injury groups subtly resembled the more punctate and discontinuous appearance of tight junctions previously observed after single blast exposure at higher severity levels.[24] We also note that

our previous work reported healthy cell morphology in bright-field micrographs and no significant cell death for injured and sham-exposed cultures after exposure to a more severe level of blast.[22]

Delayed TEER recovery after repeated blast

Recovery of TEER in cultures exposed to moderate, repeated blast injury was delayed compared with cultures exposed to a single blast. TEER of the single injury group was significantly depressed compared with age-matched shams for up to 1 day after the initial injury delivered on day 1, but was no longer significantly different by day 2 because of recovery of the monolayer (Fig. 4). Recovery of TEER in the double injury group was delayed such that TEER remained significantly lower compared with age-matched shams up to day 3 after exposure to the initial insult (Fig. 4). TEER of all cultures was monitored out to day 6 after the initial exposure, and there were no significant differences among the sham, single, or double injury groups from days 4 to 6.

Increased hydraulic conductivity after repeated blast

Hydraulic conductivity, L_p, was measured 15 to 30 min after the second moderate blast or sham injury (similar measurement time point as solute permeability and tight junction immunostaining) to quantify water flux through the BBB model. In the single injury group, L_p was slightly elevated but not significantly different compared with sham at 24 h after exposure to one mild blast—i.e., immediately after the second sham injury time point (Fig. 5). In the double injury group, L_p was significantly elevated to $2.14 \pm 0.54 \times 10^{-7}$ cm/s/cmH_2O compared with $0.83 \pm 0.18 \times 10^{-7}$ cm/s/cmH_2O in sham controls immediately after a second blast—i.e., the same measurement time point as the single injury group (Fig. 5). There was no significant difference, however, in hydraulic conductivity between the single and double injury groups. These results suggest that L_p spontaneously recovers over time similarly to the observed changes in TEER.

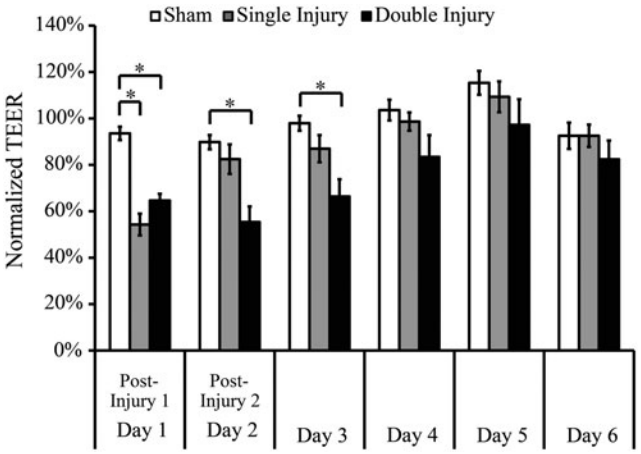

FIG. 4. Endothelial monolayers exhibited delayed recovery in transendothelial electrical resistance (TEER) after exposure to repeated moderate blast with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse. TEER in the single injury group remained significantly depressed compared with shams for up to 1 day after the initial injury. TEER of the double injury group remained significantly depressed compared with shams for 3 days after the first injury. ($*p < 0.05$; ± standard error of the mean; Sham $n = 6$; Single Injury $n = 6$; Double Injury $n = 6$).

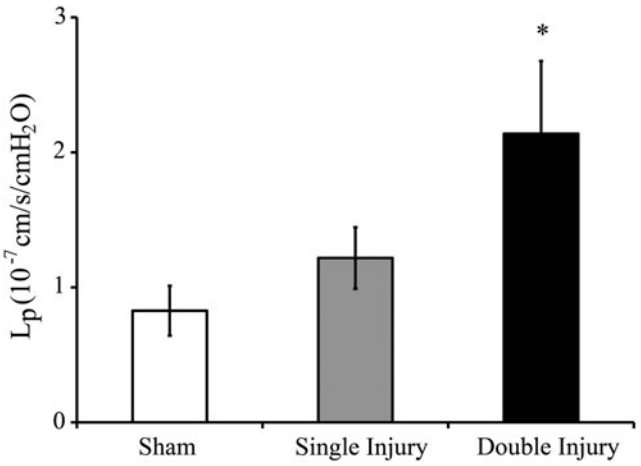

FIG. 5. Increased hydraulic conductivity after repeated blast injury in blood–brain barrier cultures. Hydraulic conductivity of endothelial monolayers exposed to consecutive moderate blasts with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse was significantly increased to $2.14 \pm 0.54 \times 10^{-7}$ cm/s/cmH_2O compared with $0.83 \pm 0.18 \times 10^{-7}$ cm/s/cmH_2O in sham controls. Hydraulic conductivity in cultures sustaining two blast injuries was not significantly different from sham controls or the single injury group when measured at the same time point (i.e., after the second sham injury). ($*p < 0.05$; ± standard error of the mean; Sham $n = 11$; Single Injury $n = 12$; Double Injury $n = 12$).

Independent effects of repeated blast on TEER with prolonged interval

To investigate the interval-specific effects of repeated blast injury in the BBB cultures, the time between injuries was extended to 72 h. After the first exposure to moderate blast, TEER of injured cultures decreased by 25 to 30% compared with sham controls (Fig. 6). Injured cultures recovered at a similar rate over time, exhibiting full recovery of TEER by day 3. After the second injury time point on day 4, TEER of the double injury group was significantly decreased by a consistent 25 to 30% in comparison with age-matched shams; this change in TEER was not significantly different than the change in TEER after the first injury (Fig. 6). The similar decrease in TEER between the double injury group and sham cultures after both the initial (day 1) and subsequent (day 4) injuries suggests that the effect of the subsequent injury on the BBB is independent (i.e., as a new single insult without residual effects from the initial exposure) given enough recovery time between the two injuries.

Discussion

Augmented brain pathology is considered to be a consequence of repetitive brain injury, and our results extend this understanding to repeated, primary blast exposure in an *in vitro* BBB model. The significant difference in TEER that we observed between cultures exposed to two moderate injuries demonstrated sustained, but not additive, effects associated with the subsequent insult (Fig. 1B). Our results add to published findings of worsened axonal and microvascular damage evoked by severity- and interval-specific repetitive TBI.[15] In an animal model of impact acceleration injury, subthreshold injuries administered in repeated fashion caused neither axonal nor vascular changes, whereas suprathreshold insults resulted in cumulative axonal damage and microvascular dysfunction.[15] It is important to note, however, that our results

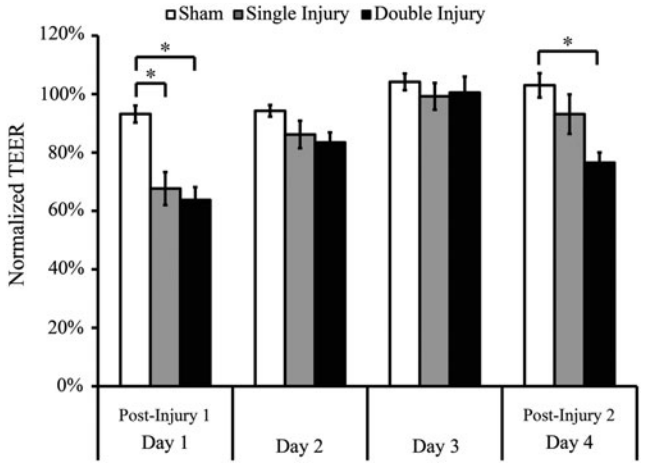

FIG. 6. Independent effects of blast on blood–brain barrier cultures over an extended 72 h interinjury interval between consecutive moderate blasts with a 402 kPa peak overpressure, 0.92 ms duration, and 118 kPa*ms impulse. The consistently similar 25 to 30% difference in transendothelial electrical resistance (TEER) between the double injury and sham groups after the first (day 1) and second (day 4) injuries suggests that the effects of repeated injuries are independent (i.e., no residual effects from the initial exposure) given a sufficiently prolonged interinjury interval. (*$p < 0.05$; ± standard error of the mean; Sham $n = 6$; Single Injury $n = 6$; Double Injury $n = 6$).

demonstrated sustained depression in TEER representing persistent injury to the BBB after multiple insults, which is distinct from additive damage to the barrier. This discrepancy may underscore fundamental differences between primary blast injury and inertial- and impact-driven injuries.

Clinically, the diagnosis of repeated mild blast exposure is challenging because of the difficulty in assessing damage using conventional neuroimaging or by impairments determined by neurobehavioral assessments.[34,35] Repeated exposure to mild explosive blast presents a significant challenge to the military health care system because of the frequency of this type of exposure, the potential for cumulative effects of multiple injuries, and the delayed onset of cognitive and behavioral impairments experienced by warfighters.[6,35,36] In neurosurgical (severe) cases of bTBI from IEDs, cerebral vasospasm was detected in a substantial number of patients who generally experienced poorer outcomes.[37,38] The constriction of blood vessels has also been confirmed experimentally in a mouse model of repeated blast-induced neurotrauma.[39] Observations of exacerbated vascular dysfunction are consistent with the second-impact syndrome reported in humans, whereby subsequent brain injuries may cause significant abnormalities including vasoparalysis and vascular engorgement.[15,21] BBB dysfunction caused by multiple exposures to bTBI, as reported in our study, may compromise the barrier's ability to maintain ion homeostasis and limit the entry of neurotoxic and inflammatory serum constituents—functions needed to preserve the health of neurons, physiologic neural signaling, and network connectivity.[40] By extending the duration of the opening of the BBB with multiple exposures, the influx of serum components and their subsequent pathophysiological effect could be increased without necessarily causing more severe BBB breakdown.

Increased BBB permeability has been reported after bTBI,[23,25,26] but it is still unknown whether repeated mild blast exposure has the potential to exacerbate leakiness of the barrier. A previous study from our laboratory demonstrated significantly increased permeability of 10 kDa dextrans through an *in vitro* BBB model after exposure to a single severe blast (571 kPa peak overpressure, 1.06 ms duration, and 186 kPa*ms impulse).[24] In other studies, permeability of the BBB was enhanced after a single primary blast exposure *in vivo* as measured by increased extravasation of sodium fluorescein (376 Da), Evans blue (70 kDa when bound to plasma albumin),[41] and IgG (approximately 150 kDa).[7,23,25,26,42]

A detailed understanding of blast-induced disruption of cerebral vascular integrity is of clinical importance because greater adhesion and infiltration of immune cells, including macrophages, can promote neurovascular inflammation and degeneration *in vivo*.[7] Interestingly, exposure to moderate blast (402 kPa) in the current study did not increase solute permeability, even after repeated exposure over a 24-h interval. These data suggest that repeated blast exposure at mild or moderate levels may establish intercellular structural voids not sufficient to permit an influx of larger solutes ($\geq$3kDa) through the barrier, but large enough to allow changes in paracellular ion-flux as previously observed in TEER measurements.

The effect of multiple blast exposures on tight junctions that critically mediate the restrictive properties of the BBB is still an area of active investigation. Previously, we demonstrated that exposure to a single, severe blast injury significantly compromised ZO-1 immunostaining in a brain endothelial monolayer.[24] Results from the current study also demonstrate that repeated blast exposure at lower intensities can significantly reduce immunostaining of the ZO-1 and claudin-5 tight junction proteins compared with sham controls; however, the degree of staining after exposure to two injuries did not significantly differ from that after a single injury.

Another investigation of exposure to a single primary blast with a 123 kPa peak overpressure (estimated 5 ms duration) in rats reported significant reductions in expression of ZO-1, claudin-5, and occludin.[7] Consistent with our results *in vitro,* the same study reported that two consecutive blast injuries delivered 24 h apart in rats reduced immunostaining for tight junction proteins compared with controls, but not compared with cultures sustaining a single blast.[7] These data strongly suggest that the effects of repeated blast on the integrity of the BBB are not associated with significantly worse tight junction damage over consecutive insults experienced with a 24 h time frame.

The time course we measured for changes in TEER suggests that repeated blast exposure delays recovery of the damaged BBB. A recent study revealed that exposure to two mild primary blast injuries with a 123 kPa peak overpressure and 4 to 5 ms duration, separated by 24 h, prolonged vascular damage as opposed to significantly exacerbating it.[7] In our BBB culture, repeated exposure to moderate blast (402 kPa) delayed spontaneous recovery of the injured monolayer. TEER of cultures exposed to a single insult remained significantly depressed for 1 day after the injury, whereas in cultures exposed to two insults, TEER remained significantly depressed for 3 days after the initial injury. We are the first to report delayed recovery of TEER after repeated, pure primary blast injury, and our time course for *in vitro* barrier recovery is reasonably similar to that observed *in vivo* at comparable blast exposure levels.[23,25] Previous investigations of BBB disruption induced by single blast exposure in small animals have reported peak IgG extravasation at 3 and 24 h post-injury, with complete resolution by 3 days after exposure.[23,25] Future work will examine the cellular mechanisms responsible for BBB recovery, but it has been suggested that shock wave–induced injury to the cerebral vasculature gradually diminishes over time potentially because of repair

processes that help mitigate subsequent damage from consecutive blast exposures.[5,7]

Brain edema has been reported to be a characteristic outcome of bTBI.[43,44] In the current investigation, hydraulic conductivity in our *in vitro* BBB model was significantly increased immediately after a second injury compared with sham controls. Similar to the time course of TEER, hydraulic conductivity recovered 24 h after a single injury to lower pre-injury levels. Although in this study we did not measure changes in water flux acutely after the initial injury time point, our previous study describes changes in hydraulic conductivity in response to a single blast over a range of severity levels.[24]

These results are supported by previous findings that mice exposed to three consecutive blast injuries with an estimated 142 kPa peak overpressure (unreported duration) separated by 1 and 30 min intervals had significantly increased brain water content compared with sham controls.[13] Changes in brain edema were detected 4 h post-injury and were no longer apparent at 24 and 48 h after blast exposure.[13] Multiple exposures to low-level blasts in rats and pigs were associated with increased intracranial pressure, which may be attributed to edema, hemorrhage, and BBB damage.[11–13,45] The development of cerebral edema around the vasculature is often associated with enhanced expression of the water channel protein, aquaporin-4 (AQP4), in the perivascular region after impact- and blast-induced injuries to the head.[7,46] Further studies are warranted to identify the underlying mechanisms responsible for increased water flux through the barrier after blast exposure.

An important outcome of the current study is that extension of the interval between the initial and subsequent blast injuries from 24 to 72 h allowed TEER to fully recover before delivery of the subsequent insult. Microvascular restoration in the interinjury interval has been reported by others using an impact-driven repetitive TBI model.[15] After the second injury in our study, TEER decreased by 25 to 30% compared with sham controls, which was equivalent to the change in TEER observed acutely after the first blast. Together, these results suggest that the BBB response to repeated blasts may be independent; that is, there is no evidence of residual effects across consecutive exposures given a sufficient delay between injuries.

The ability to ameliorate the persistent burden of BBB disruption is supported by published studies reporting the reduction or complete elimination of axonal damage, vascular dysfunction, and compromises in cerebral metabolism when the interval between consecutive injuries was sufficiently extended.[15,17,19,47] Overall, such findings support the existence of a window of heightened vulnerability of the BBB to repetitive primary blast injury, which holds implications for a minimum mandatory rest period (several days) for blast-injured service members before returning to duty.

We note that our results were from an isolated component of the BBB and do not preclude the possibility that repeated primary blast may have cumulative and direct effects on other components of the brain, such as neurons or glia or on their interactions. *In vivo*, the BBB is a heterogeneous structure consisting of brain capillary endothelial cells (e.g. bEnd.3 cells), which together with astrocytes, pericytes, microglia, neurons, and the extracellular matrix, make up the neurovascular unit.[48,49] After brain injury, such as TBI, the physiological interactions among the various cellular components of this unit are significantly altered. Such changes can lead to abnormal tight junction protein expression, an inflammatory response mediated by astrocytes and microglia, and altered neuronal activity, among others.[49] Consistent with this, exposure to a pure shock wave in rats induced free radical-generating enzymes, oxidative damage markers, a reduction in tight junction proteins, BBB leakage, upregulation of perivascular matrix metalloproteinases, and an increase in AQP4 expression in astrocytes, ultimately leading to neuroinflammation.[7]

While our previous work describes in greater detail the advantages and limitations of using an *in vitro* model to study the effects of primary blast on the BBB,[24] the need to better understand the influence of other cell types of the neurovascular unit on BBB dysfunction after blast injury motivates further study *in vivo*.

Comparison of our results with those published in the literature may be limited by variability in the blast parameters tested among different experimental blast injury models. For example, one recent investigation of bTBI demonstrated BBB breakdown by the presence of focal lesions after exposure to low-impulse primary blast with short durations on the order of microseconds,[26] whereas typical free-field explosions have durations spanning several milliseconds.[50] BBB breakdown in animals has also been demonstrated in studies after exposure to lower levels of peak overpressure, but longer durations of approximately 4 to 5 ms.[7,23,25] In addition, despite efforts to restrain head motion during blast exposure, it is difficult to completely rule out possible contributions of concomitant inertial head injuries in the absence of high-speed video recordings.[12,13,23,25]

A previous investigation highlighted that the effects of pure primary blast exposure could be vastly different from those of blast-induced inertial loading of the head, because neurological deficits disappeared when the head was immobilized.[51] As reported previously, we have used high-speed video analysis (data not shown) to confirm the absence of gross movement of our *in vitro* BBB cultures during primary blast exposure.[24]

An advantage of our blast injury model is the ability to study damage to an *in vitro* model of the BBB purely as a result of primary blast injury, in the absence of confounding contributions from inertial loading mechanisms.[22,24] A previous study using finite element modeling to simulate biomechanical parameters of our *in vitro* blast injury model predicted tissue strain rates less than $80\ s^{-1}$ and principal strains not exceeding 5%,[27,52] which are levels significantly below the thresholds for functional deficits and axonal death reported for living brain tissue.[53,54] Together, these previous investigations provide further support that the injuries modeled using our methodology are from overpressure-loading and not strain-loading in the sample.

A limitation to consider is that our repeated blast paradigm consisted of only two consecutive injuries, so caution should be exercised when extending our results to situations with more blasts.

Conclusion

This study contributes to a growing body of work indicating that brain microvascular dysfunction can result from repetitive injuries at specific severity levels applied over a well-defined time frame. Our results do not support the hypothesis that damage to the BBB by repeated blasts is cumulative, at least for two blast exposures delivered 24 h apart from one another. Results of our investigation are in strong agreement with studies reporting that, despite dramatic pathological changes observed after a single injury, multiple exposures delivered within a short time frame delay recovery, rather than cause additive damage to the barrier.[7] By extending the interval between injuries from 24 to 72 h, the BBB fully recovered to pre-injury levels before experiencing similar damage after the second injury as that observed after the first, together demonstrating

independent effects of multiple injuries given a sufficiently prolonged interinjury interval. Future work will examine the cellular and molecular mechanisms that underpin the observed changes in BBB integrity and function.

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Author Disclosure Statement

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References

1. Ahlers, S.T., Vasserman-Stokes, E., Shaughness, M.C., Hall, A.A., Shear, D.A., Chavko, M., McCarron, R.M., and Stone, J.R. (2012). Assessment of the effects of acute and repeated exposure to blast overpressure in rodents: toward a greater understanding of blast and the potential ramifications for injury in humans exposed to blast. Front. Neurol. 3, 32.
2. Warden, D. (2006). Military TBI during the Iraq and Afghanistan wars. J. Head Trauma Rehabil. 21, 398–402.
3. Hoge, C.W., McGurk, D., Thomas, J.L., Cox, A.L., Engel, C.C., and Castro, C.A. (2008). Mild traumatic brain injury in U.S. Soldiers returning from Iraq. N. Engl. J. Med. 358, 453–463.
4. Elder, G.A., and Cristian, A. (2009). Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care. Mt. Sinai J. Med. 76, 111–118.
5. Kamnaksh, A., Kwon, S.K., Kovesdi, E., Ahmed, F., Barry, E.S., Grunberg, N.E., Long, J., and Agoston, D. (2012). Neurobehavioral, cellular, and molecular consequences of single and multiple mild blast exposure. Electrophoresis 33, 3680–3692.
6. Rosenfeld, J.V., and Ford, N.L. (2010). Bomb blast, mild traumatic brain injury and psychiatric morbidity: a review. Injury 41, 437–443.
7. Abdul-Muneer, P.M., Schuetz, H., Wang, F., Skotak, M., Jones, J., Gorantla, S., Zimmerman, M.C., Chandra, N., and Haorah, J. (2013). Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. Free Radic. Biol. Med. 60, 282–291.
8. Trudeau, D.L., Anderson, J., Hansen, L.M., Shagalov, D.N., Schmoller, J., Nugent, S., and Barton, S. (1998). Findings of mild traumatic brain injury in combat veterans with PTSD and a history of blast concussion. J. Neuropsychiatry Clin. Neurosci. 10, 308–313.
9. Blennow, K., Jonsson, M., Andreasen, N., Rosengren, L., Wallin, A., Hellstrom, P.A., and Zetterberg, H. (2011). No neurochemical evidence of brain injury after blast overpressure by repeated explosions or firing heavy weapons. Acta Neurol. Scand. 123, 245–251.
10. Kane, M.J., Angoa-Perez, M., Briggs, D.I., Viano, D.C., Kreipke, C.W., and Kuhn, D.M. (2012). A mouse model of human repetitive mild traumatic brain injury. J. Neurosci. Methods 203, 41–49.
11. Saljo, A., Mayorga, M., Bolouri, H., Svensson, B., and Hamberger, A. (2011). Mechanisms and pathophysiology of the low-level blast brain injury in animal models. Neuroimage 54, Suppl 1, S83–S88.
12. Saljo, A., Svensson, B., Mayorga, M., Hamberger, A., and Bolouri, H. (2009). Low-level blasts raise intracranial pressure and impair cognitive function in rats. J. Neurotrauma 26, 1345–1352.
13. Wang, Y., Wei, Y., Oguntayo, S., Wilkins, W., Arun, P., Valiyaveettil, M., Song, J., Long, J.B., and Nambiar, M.P. (2011). Tightly coupled repetitive blast-induced traumatic brain injury: development and characterization in mice. J. Neurotrauma 28, 2171–2183.
14. Elder, G.A., Dorr, N.P., De Gasperi, R., Gama Sosa, M.A., Shaughness, M.C., Maudlin-Jeronimo, E., Hall, A.A., McCarron, R.M., and Ahlers, S.T. (2012). Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. J. Neurotrauma 29, 2564–2575.
15. Fujita, M., Wei, E.P., and Povlishock, J.T. (2012). Intensity- and interval-specific repetitive traumatic brain injury can evoke both axonal and microvascular damage. J. Neurotrauma 29, 2172–2180.
16. Friess, S.H., Ichord, R.N., Ralston, J., Ryall, K., Helfaer, M.A., Smith, C., and Margulies, S.S. (2009). Repeated traumatic brain injury affects composite cognitive function in piglets. J. Neurotrauma 26, 1111–1121.
17. Longhi, L., Saatman, K.E., Fujimoto, S., Raghupathi, R., Meaney, D.F., Davis, J., McMillan, B.S., Conte, V., Laurer, H.L., Stein, S., Stocchetti, N., and McIntosh, T.K. (2005). Temporal window of vulnerability to repetitive experimental concussive brain injury. Neurosurgery 56, 364–374.
18. Shitaka, Y., Tran, H.T., Bennett, R.E., Sanchez, L., Levy, M.A., Dikranian, K., and Brody, D.L. (2011). Repetitive closed-skull traumatic brain injury in mice causes persistent multifocal axonal injury and microglial reactivity. J. Neuropathol. Exp. Neurol. 70, 551–567.
19. Prins, M.L., Alexander, D., Giza, C.C., and Hovda, D.A. (2013). Repeated mild traumatic brain injury: mechanisms of cerebral vulnerability. J. Neurotrauma 30, 30–38.
20. Peskind, E.R., Petrie, E.C., Cross, D.J., Pagulayan, K., McCraw, K., Hoff, D., Hart, K., Yu, C.E., Raskind, M.A., Cook, D.G., and Minoshima, S. (2011). Cerebrocerebellar hypometabolism associated with repetitive blast exposure mild traumatic brain injury in 12 Iraq war Veterans with persistent post-concussive symptoms. Neuroimage 54, Suppl 1, S76–S82.
21. Cantu, R.C., and Gean, A.D. (2010). Second-impact syndrome and a small subdural hematoma: an uncommon catastrophic result of repetitive head injury with a characteristic imaging appearance. J. Neurotrauma 27, 1557–1564.
22. Effgen, G.B., Hue, C.D., Vogel, E., 3rd, Panzer, M.B., Meaney, D.F., Bass, C.R., and Morrison, B., 3rd. (2012). A multiscale approach to blast neurotrauma modeling: Part II: methodology for inducing blast injury to in vitro models. Front. Neurol. 3, 23.
23. Garman, R.H., Jenkins, L.W., Switzer, R.C., 3rd, Bauman, R.A., Tong, L.C., Swauger, P.V., Parks, S.A., Ritzel, D.V., Dixon, C.E., Clark, R.S., Bayir, H., Kagan, V., Jackson, E.K., and Kochanek, P.M. (2011). Blast exposure in rats with body shielding is characterized primarily by diffuse axonal injury. J. Neurotrauma 28, 947–959.
24. Hue, C.D., Cao, S., Haider, S.F., Vo, K.V., Effgen, G.B., Vogel III, E., Panzer, M.B., Bass, C.D., Meaney, D., and Morrison III, B. (2013). Blood-brain barrier dysfunction after primary blast injury in vitro. J. Neurotrauma 30,1652–1663.
25. Readnower, R.D., Chavko, M., Adeeb, S., Conroy, M.D., Pauly, J.R., McCarron, R.M., and Sullivan, P.G. (2010). Increase in blood-brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury. J. Neurosci. Res. 88, 3530–3539.
26. Yeoh, S., Bell, E.D., and Monson, K.L. (2013). Distribution of blood-brain barrier disruption in primary blast injury. Ann. Biomed. Eng. 41, 2206–2214.
27. Panzer, M.B., Matthews, K.A., Yu, A.W., Morrison, B., 3rd, Meaney, D.F., and Bass, C.R. (2012). A multiscale approach to blast neurotrauma modeling: Part I - Development of novel test devices for in vivo and in vitro blast injury models. Front. Neurol. 3, 46.
28. Li, G., Simon, M.J., Cancel, L.M., Shi, Z.D., Ji, X., Tarbell, J.M., Morrison, B., 3rd, and Fu, B.M. (2010). Permeability of endothelial and astrocyte cocultures: in vitro blood–brain barrier models for drug delivery studies. Ann. Biomed. Eng. 38, 2499–2511.
29. Simon, M.J., Kang, W.H., Gao, S., Banta, S., and Morrison, B., 3rd (2011). TAT is not capable of transcellular delivery across an intact endothelial monolayer in vitro. Ann. Biomed. Eng. 39, 394–401.
30. Madara, J.L. (1998). Regulation of the movement of solutes across tight junctions. Ann. Rev. Physiol. 60, 143–159.
31. Brown, R.C., Morris, A.P., and O'Neil, R.G. (2007). Tight junction protein expression and barrier properties of immortalized mouse brain microvessel endothelial cells. Brain Res. 1130, 17–30.
32. Sill, H.W., Chang, Y.S., Artman, J.R., Frangos, J.A., Hollis, T.M., and Tarbell, J.M. (1995). Shear stress increases hydraulic conductivity of cultured endothelial monolayers. Am. J. Physiol. 268, H535–H543.
33. Omidi, Y., Campbell, L., Barar, J., Connell, D., Akhtar, S., and Gumbleton, M. (2003). Evaluation of the immortalised mouse brain

capillary endothelial cell line, b.End3, as an in vitro blood–brain barrier model for drug uptake and transport studies. Brain Res. 990, 95–112.
34. Belanger, H.G., Vanderploeg, R.D., Curtiss, G., and Warden, D.L. (2007). Recent neuroimaging techniques in mild traumatic brain injury. J. Neuropsychiatry Clin. Neurosci. 19, 5–20.
35. Brenner, L.A., Vanderploeg, R.D., and Terrio, H. (2009). Assessment and diagnosis of mild traumatic brain injury, posttraumatic stress disorder, and other polytrauma conditions: burden of adversity hypothesis. Rehabil. Psychol 54, 239–246.
36. Kamnaksh, A., Kovesdi, E., Kwon, S.K., Wingo, D., Ahmed, F., Grunberg, N.E., Long, J., and Agoston, D.V. (2011). Factors affecting blast traumatic brain injury. J. Neurotrauma 28, 2145–2153.
37. Armonda, R.A., Bell, R.S., Vo, A.H., Ling, G., DeGraba, T.J., Crandall, B., Ecklund, J., and Campbell, W.W. (2006). Wartime traumatic cerebral vasospasm: recent recview of combat casualties. Neurosurgery 59, 1215–1225.
38. Long, J.B., Bentley, T.L., Wessner, K.A., Cerone, C., Sweeney, S., and Bauman, R.A. (2009). Blast overpressure in rats: recreating a battlefield injury in the laboratory. J. Neurotrauma 26, 827–840.
39. Valiyaveettil, M., Alamneh, Y., Wang, Y., Arun, P., Oguntayo, S., Wei, Y., Long, J.B., and Nambiar, M.P. (2013). Contribution of systemic factors in the pathophysiology of repeated blast-induced neurotrauma. Neurosci. Lett. 539, 1–6.
40. Abbott, N.J., and Friedman, A. (2012). Overview and introduction: the blood-brain barrier in health and disease. Epilepsia 53, Suppl 6, 1–6.
41. Greish, K. (2007). Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines. J. Drug Target. 15, 457–464.
42. Kuehn, R., Simard, P.F., Driscoll, I., Keledjian, K., Ivanova, S., Tosun, C., Williams, A., Bochicchio, G., Gerzanich, V., and Simard, J.M. (2011). Rodent model of direct cranial blast injury. J. Neurotrauma 28, 2155–2169.
43. Cernak, I., and Noble-Haeusslein, L.J. (2010). Traumatic brain injury: an overview of pathobiology with emphasis on military populations. J. Cereb. Blood Flow Metab. 30, 255–266.
44. Mac Donald, C.L., Johnson, A.M., Cooper, D., Nelson, E.C., Werner, N.J., Shimony, J.S., Snyder, A.Z., Raichle, M.E., Witherow, J.R., Fang, R., Flaherty, S.F., and Brody, D.L. (2011). Detection of blast-related traumatic brain injury in U.S. military personnel. N. Engl. J. Med. 364, 2091–2100.
45. Saljo, A., Arrhen, F., Bolouri, H., Mayorga, M., and Hamberger, A. (2008). Neuropathology and pressure in the pig brain resulting from low-impulse noise exposure. J. Neurotrauma 25, 1397–1406.
46. Higashida, T., Kreipke, C.W., Rafols, J.A., Peng, C., Schafer, S., Schafer, P., Ding, J.Y., Dornbos, D., 3rd, Li, X., Guthikonda, M., Rossi, N.F., and Ding, Y. (2011). The role of hypoxia-inducible factor-1alpha, aquaporin-4, and matrix metalloproteinase-9 in blood-brain barrier disruption and brain edema after traumatic brain injury. J. Neurosurg. 114, 92–101.
47. Vagnozzi, R., Signoretti, S., Tavazzi, B., Floris, R., Ludovici, A., Marziali, S., Tarascio, G., Amorini, A.M., Di Pietro, V., Delfini, R., and Lazzarino, G. (2008). Temporal window of metabolic brain vulnerability to concussion: a pilot 1H-magnetic resonance spectroscopic study in concussed athletes—part III. Neurosurgery 62, 1286–1296.
48. Gumbleton, M., and Audus, K.L. (2001). Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. J. Pharm. Sci. 90, 1681–1698.
49. Shlosberg, D., Benifla, M., Kaufer, D., and Friedman, A. (2010). Blood–brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat. Rev. Neurol. 6, 393–403.
50. Bauman, R.A., Ling, G., Tong, L., Januszkiewicz, A., Agoston, D., Delanerolle, N., Kim, Y., Ritzel, D., Bell, R., Ecklund, J., Armonda, R., Bandak, F., and Parks, S. (2009). An introductory characterization of a combat-casualty-care relevant swine model of closed head injury resulting from exposure to explosive blast. J. Neurotrauma 26, 841–860.
51. Goldstein, L.E., Fisher, A.M., Tagge, C.A., Zhang, X.L., Velisek, L., Sullivan, J.A., Upreti, C., Kracht, J.M., Ericsson, M., Wojnarowicz, M.W., Goletiani, C.J., Maglakelidze, G.M., Casey, N., Moncaster, J.A., Minaeva, O., Moir, R.D., Nowinski, C.J., Stern, R.A., Cantu, R.C., Geiling, J., Blusztajn, J.K., Wolozin, B.L., Ikezu, T., Stein, T.D., Budson, A.E., Kowall, N.W., Chargin, D., Sharon, A., Saman, S., Hall, G.F., Moss, W.C., Cleveland, R.O., Tanzi, R.E., Stanton, P.K., and McKee, A.C. (2012). Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci. Transl. Med. 4, 134ra160.
52. Panzer, M.B., Myers, B.S., Capehart, B.P., and Bass, C.R. (2012). Development of a finite element model for blast brain injury and the effects of CSF cavitation. Ann. Biomed. Eng. 40, 1530–1544.
53. Bain, A.C., and Meaney, D.F. (2000). Tissue-level thresholds for axonal damage in an experimental model of central nervous system white matter injury. J. Biomech. Eng. 122, 615–622.
54. Morrison, B., 3rd, Cater, H.L., Wang, C.C., Thomas, F.C., Hung, C.T., Ateshian, G.A., and Sundstrom, L.E. (2003). A tissue level tolerance criterion for living brain developed with an in vitro model of traumatic mechanical loading. Stapp Car Crash J. 47, 93–105.

Address correspondence to:
Barclay Morrison III, PhD
Biomedical Engineering
Columbia University
351 Engineering Terrace, MC8904
1210 Amsterdam Avenue
New York, NY 10027

E-mail: bm2119@columbia.edu