A COMPARATIVE ANALYSIS OF BLAST-INDUCED NEUROTRAUMA AND BLUNT TRAUMATIC BRAIN INJURY REVEALS SIGNIFICANT DIFFERENCES IN INJURY MECHANISMS

UPOREDNA ANALIZA BLAST NEUROTRAUME I TUPE POVREDE MOZGA OTKRIVA ZNAČAJNE RAZLIKE U MEHANIZMU POVREĐIVANJA

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Abstract

Blast-induced neurotrauma (BINT) is caused by complex physical environment generated by an explosion and diverse effects of the resulting blast. Clinical experience suggests specific blast-body-nervous system interactions, and resulting complex cellular and molecular mechanisms that can lead to long-term neurological deficits. While the pathobiology of BINT is not fully understood, the growing number of servicemen experiencing significant neurological deficits necessitates the development of reliable and injury-specific diagnostic and therapeutic measures. Aiming to identify the similarities and differences between BINT and blunt impact traumatic brain injury, we used well-standardized corresponding mouse models to analyze physiological (arterial blood oxygen saturation, heart rate, respiratory rate, and pulse distention), functional (motor performance, exploratory activity), and molecular (glial-acidic fibrillary protein) alterations that occurred 30 days after injury. Our results demonstrate that the generalizable consequences of a brain insult, such as decrease in motor performance and exploratory activity as well as stimulation of astrocytes, have differing temporal profiles, suggesting injury-specificity that should be taken into account when developing diagnostic and differential diagnostic methods.
INTRODUCTION

Traumatic brain injuries caused by explosions have progressively increasing importance for both military and civilian populations. Blast induced neurotrauma (BINT) represented the most frequent type of traumatic brain injury (TBI) in the military during operations abroad in Iraq and Afghanistan(1). Accumulating experimental data and clinical evidence shows that blast waves can cause brain injury with or without penetrating wounds of the head. This is caused by a series of effects such as(1) primary blast, or the shock wave itself;(2) secondary blast, where fragments of debris are propelled by the explosion;(3) tertiary blast, which is the acceleration/deceleration of whole or part of the body by a blast wind (Fig. 1); and 4) quaternary, where flash burns are a consequence of the transient but intense heat of the explosion(2, 3). Unfortunately, BINT is not only a military problem: a high proportion of diffuse brain injury due to blasts and relative to all other types of injuries has been observed among civilians both during the wartime(4) and in a peacetime setting(5).

There are numerous controversial opinions about the cause of neurological deficits that develop after being exposed to blast. These opinions range from the belief that blast-induced mild traumatic brain injury is a mere concussion, and as such the nature of its symptoms are those of a temporary post-concussive syndrome(6), to the belief that blast-induced neurological deficits are caused by unique interactions of systemic, local, biomechanical, and cerebral responses to blast(7-9). Despite concentrated efforts to clarify the molecular mechanisms underlying BINT, definitive diagnosis and specific evidence-based therapy have yet to be developed for preventing and/or reducing the detrimental effects of blast exposure on the brain.

Blunt head trauma (bTBI) is the leading cause of death and disability in civilian population under 40 years of age. Indeed, each year, an estimated 1.5 million Americans sustain a TBI. As a result of these injuries, 50,000 people die, 230,000 people are hospitalized and survive, and an estimated 80,000-90,000 people experience the onset of long-term disability(10). Despite the enormity of this public health problem, no effective treatment exists.

Taking into account the complexity of the physical environment generated by explosion as well as the involvement of multiple organs and organ systems responding to blast exposure, we hypothesize that the injury response profile in BINT is significantly different than in bTBI.

The main goal of this study was to compare physiological, neurological, and molecular mechanisms of BINT and bTBI using well-defined experimental models, bearing in mind that establishing similarities and differences in the pathobiology of resulting functional deficits would significantly contribute to developing insult-specific diagnostic measures.

MATERIAL AND METHODS

All protocols involving the use of animals complied with the NIH Guide and Care and Uses of Laboratory Animals published by NIH (DHEW publication NIH 85-23-2985) and were approved by the Johns Hopkins University Animal Use Committee (protocol, MO0M1O2).

Injury Induction

Mice (C57/B16; male; 3-4 months; 25.22 ±1.96 g; Jackson Laboratories; n=309 total) had access to food and water ad libitum. Animals were anesthetized with 4% isoflurane evaporated in a gas mixture containing 30% oxygen/70% nitrous oxide and applied through a nose-mask. The animals were allowed to breathe spontaneously without tracheal intubation. Immediately after injury (either BINT or bTBI), anesthesia was discontinued and the animals were maintained on room air alone without resuscitation. Acute neurological recovery was assessed in all mice by recording indicators of sensorimotor function: recovery of hind paw-extension following the gradual application of pressure and the latency to recovery of the righting reflex. The lethality rate was established at 24 h post-trauma.

BINT

The shock tube developed by the Johns Hopkins University Applied Physics Laboratory (JHU/APL) is a modular, multi-chamber device capable of reproducing complex shock wave signatures seen in theater and tailoring pressure wave signatures(11). The single-driver, compressed-helium operated shock tube-system generated dynamic over-pressure loading conditions necessary to induce mild, moderate, and severe levels of lethality in mice as previously described(11). Briefly, the mouse was mounted in supine position to the animal holder, with the neck, head, torso, and abdomen of the animal firmly fixed to avoid any movement, thus tertiary blast effects. The animal holder was secured and positioned inside the driven section of the shock tube so that the animal was at 53 cm (20.87 inch) upstream from the driven section opening, ensuring that the only a well-formed incident shock wave loaded the animal and that potential raf-ections from the tube opening were minimized. Mild blast injury (LD5) was induced by a shock wave of 183 ± 14 kPa; moderate blast injury (LD37) was generated by 213 ± 17 kPa; whereas a shock wave of 295 ± 32 kPa lead to severe blast injury (LD53).

bTBI

The skull of the anesthetized mouse was exposed with a single sharp cut in a longitudinal manner (2.0-2.5 cm) to detect the correct location and to mark the area to be impacted. Then the mouse was placed onto the mobile platform of the bTBI device and the tip of the metal rod of the device slowly advanced onto exposed mouse skull to determine the precise area of impact. Upon satisfactory positioning of the blunt tip, bilateral blocks under the mobile platform were pushed to ensure stable positioning for the traumatic impact and the rod was retracted to its proper position, i.e., falling height determined by the desired severity of the injury (12). The injury severity was established based on the distance from which the metal rod was dropped on the middle of the skull; thus, mild, moderate, and severe bTBI with lethality rates comparable to those induced by BINT were induced by drops from 1.5 cm, 2 cm, and 3 cm distances, respectively. Trauma was applied by pressing the pedal of the trauma apparatus. Following impact, the rod was retracted immediately to prevent unwarranted secondary trauma. Finally, the skin was sutured to close the skull and the animals were returned to their cages and given the proper food and water supply.
Physiological Parameters

Body weight (BW) measurements were performed before, and at 3, 5, 7, 10, 14, 18, 21, 25, and 30 days post-injury.

Multiple vital signs were measured non-invasively using the MouseOx Pulse Oxymeter (Starr Life Sciences Corp., Oakmont, PA) before injury; at 10 minutes (“immediately”); and at 7, 14, 21, and 30 days post-injury. The sensor clip of the device was wrapped around the mouse neck, and vital signs were measured non-invasively. Measured parameters included arterial blood oxygen saturation included: 1) the oxygen saturation of hemoglobin reported after each heart-beat; 2) pulse rate, i.e., heart rate (HR) with a measurement range of 90 to 900 beat per minute (bpm); 3) respiratory rate (RR) reported every 1.7 seconds where the value reported is the result of a moving range of the rate of breathing for the 10 previous breath rate measurements; and 4) pulse distention (PD), i.e., a measurement of the distention of the arterial blood vessels (carotid artery) between the sensors due to a cardiac pulse and used as a non-invasive indicator of a blood flow.

Neurological Outcome

Motor Function Test

Using the rotarod test, motor scoring was performed before, and at 1, 2, 3, 5, 7, 10, 14, 21, and 30 days after injury. This test is one of the most sensitive tests to detect motor deficits in rodent brain injury\(^{(13)}\). Briefly, the animals were placed on a rotarod device (Panlab Rota-Rods LE 8200, Harvard Apparatus, Holliston, MA), and permitted to explore the rotarod for 2 minutes without rotation. Then the mice experienced a period of slow acceleration of the drum to 14 rotations per minute (rpm). The mice were subjected to 2-minute training trials for seven consecutive days before injury. The rotarod test was performed by first placing the mice on the rotating drum (25 rpm) for 2 minutes and then measuring the length of time each animal was able to maintain its balance walking on top of the drum.

Behavioral Test

Spontaneous exploratory behavior after injury was assessed using the open field test\(^{(14)}\). This test was conducted in a white, opaque rectangular polypropylene arena measuring 45 cm x 45 cm x 40 cm with a lamp providing 550 lux illumination positioned 30 cm above the arena. Each mouse...
was placed individually into the center of the arena and allowed to roam freely in the box. The animal’s movement was recorded over a 5-min period by using a video camera. The tests were performed before injury, and on days 1, 2, 3, 5, 7, 10, 14, 21, and 30 post-injury. The tracking program AnyMaze® (Stoelting, Wood Dale, IL) was used to calculate both the frequency and duration of a range of behavior. The parameters included distance covered (m); mean speed of movement (m/s); number of rearing as a measure of exploratory activity; number of center entries; time spent in the center; and time spent immobile (s).

**Semi-Quantitative RT-PCR**

To measure activation of the glial fibrillary acidic protein (GFAP), an indicator of astrocyte reactivity associated with glial scar formation in the brain, subsets of mice exposed to BINT or bTBI were anesthetized with 2-2-2-tribromoethanol (125 mg/kg, Sigma), and euthanized at 1, 3, 7, 14, and 30 days after mild or moderate injury. Naïve mice (n = 2) were sacrificed immediately after reaching the surgical level of anesthesia.

Total RNA was isolated from the dissected hippocampus and brainstem at different time points followed by BINT or bTBI (Total RNA Miniprep kit; La Jolla, CA). Total RNA (0.5 µg) was separated by electrophoresis on a 1.2% agarose, 2.2 M formaldehyde gel to evaluate the quality of RNA samples. For cDNA synthesis, 5 µg of total RNA was reverse transcribed using Superscript (GIBCO BRL) and oligo (dT)-primer. The resulting cDNA was amplified by PCR using sense and antisense primers. The amount of synthesized cDNA was normalized by PCR using primers specific to ribosomal protein (RPL19). PCR reactions were performed using the Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems Lt., Carlsbad, CA). Each PCR reaction was repeated at least twice. The thermal cycling parameters were as follows: 1 min 30 sec at 94 °C followed by 30 cycles of 30 sec at 94 °C, 1 min 30 sec at 59 °C, 1 min at 72 °C, and final incubation for 5 min at 72 °C. PCR reaction products were analyzed by agarose gel-electrophoresis. After adjustment of cDNA concentration for each individual sample from control and experimental animals, relative abundance of mRNA for the selected gene was estimated based on the intensity of cDNA bands using Quantity One 1-D Analysis Software (BioRad, Lab., Inc. Hercules, CA).

**Statistical Analysis**

Statistical analysis was performed using Sigma Stat 2.03 (SPSS, Chicago, IL, USA) or SPSS 15.0 for Windows (SPSS). All continuous data are expressed as mean ± SD and were analyzed by repeated measures analysis of variance followed by individual Student-Neuman-Keuls test. Gene expression measurements were analyzed by two-way ANOVA and P-values for overall effects of time are reported in full to three decimal places. Tukey’s post hoc test was used for comparisons between and within individual injury groups and time points, reported as P < 0.05 or < 0.001. Statistically significance was considered at the 5% level.
Pulse Distention

The pulse distention measurements that gave insight into blood flow alterations in the carotid arteries, demonstrated significant reduction in both mild and moderate BINT groups as compared to the pre-injury values and the bTBI groups (Fig. 3C). Increased values of PD were observed in animals with mild BINT at 14, 21, and 30 days post-trauma. On the other hand, PD was mainly unchanged in the bTBI groups, other than a significant (p<0.001) increase in animals with moderate bTBI 21 days after the insult.

Respiratory Rate

In mice with mild bTBI, the respiratory rate (RR) (Fig. 3D) was significantly increased (p<0.01) immediately after impact, and then closed to the normal values until the end of the observation period. In the moderate bTBI group, RR gradually increased reaching the peak at day 21 post-injury (p<0.01) as compared to the pre-injury level, followed by a normalization on day 30. In comparison, there was a significant drop (p<0.001 compared to pre-exposure levels) in the RR measured in both mild and moderate BINT immediately after blast. Later, RR was significantly increased in the moderate BINT group at 14 and 30 days post-exposure, as compared to both pre-injury levels and the moderate bTBI group.

Functional Alterations

Motor Function

Figure 4A shows motor performance in animals exposed to bTBI or BINT. Both the mild and moderate bTBI groups demonstrated a significant decline in rotarod performance during the first two weeks after the impact. While the motor performance was normalized in animals with mild BINT after day 14 post-trauma, the mice with moderate bTBI manifested continued reduction in motor performance at the end of the 30-day observation period. Nevertheless, although animals with BINT showed comparable temporal profile of motor function alterations, their motor deficit was significantly more pronounced than in the bTBI groups.

Exploratory Activity

In the open field spontaneous activity test, the both the blunt impact or blast exposure significantly reduced the animals’ exploratory activity, measured by time spent in rearing over a 5-min period (Fig. 4B). The reduced interest toward environment was persistent in all animals throughout the entire post-traumatic period, and showed dose-dependency to injury severity. At the end of the one-month observation period, the decrease in exploratory activity was most prominent in animals with moderate BINT.

Changes in GFAP Expression

Figure 5 provides information on the expression levels of the GFAP, chosen as an indicator of astrocyte activation \((15-17)\), in the brainstem (Fig. 5A) and hippocampus (Fig. 5B) of
 naïve, sham, and injured animals at 1, 3, 7, 14, and 30 days post-trauma. Similar to the functional outcome measures, changes in GFAP gene expression showed a multi-phase pattern.

**Brainstem**

Significant GFAP upregulation was observed in animals with moderate BINT as well as in the moderate bTBI group throughout the entire 30-day follow-up period (Fig. 5A). Nevertheless, the temporal profile of the changes was different in the BINT groups as compared to mice with bTBI. While the highest GFAP signals were measured in moderate BINT group at 3 and 30 days post-exposure, in animals with moderate bTBI the peak GFAP values were seen at 14 days after the insult. Most importantly, while the GFAP expression showed a declining trend in animals with blunt head trauma, it remained significantly increased in animals with blast injuries one month after the insult.

**Hippocampus**

The GFAP changes in hippocampus were somewhat different in all experimental groups as compared to those measured in the brainstem (Fig. 5B). Nevertheless, similar to the brainstem measurements, in the group of animals with BINT, the highest upregulation was observed in animals with moderate BINT at day 3 and 30 post-exposure. Blunt head trauma also induced significant upregulation of GFAP, with the peak values in moderately injured animals on day 7 after the insult. It is noteworthy that comparable to the brainstem, the GFAP expression changes suggested trend toward normalization in animals with mild BINT, mild bTBI, or moderate BINT, but remained significantly increased in animals with moderate BINT.

**DISCUSSION**

Throughout current military actions, explosive devices have become more powerful, their detonation systems more creative, and their additives more devastating. As a continuing threat to both military troops and civilians, blast injuries, especially BINT, are called the signature wound of the war in Iraq. In both civilian and military environments, exposure to a blast may cause instant death, injuries with immediate manifestation of symptoms, and latent injuries that are initiated at the time of exposure but manifest over a period of hours, months, or even years (9). The improved interceptive properties of body armor have increased the number of surviving soldiers by protecting them from penetrating injuries. Nevertheless, in parallel with this increased survival-rate, the number of victims with severe debilitating long-term
consequences, not seen before, is also increased. While body armor affords protection from shrapnel and projectiles, it also provides an improved contact surface for shock front-body interaction and energy transfer, potentially increasing the risk of injury due to the primary blast wave. Further, body armor may serve as a reflecting surface that can concentrate the power of the explosion as the blast wave reflects off the armor front and back. Moreover, in-theater soldiers as well as some non-combatant military professionals are subjected to repeated low-level blast exposures during their daily activities and/or training. The accumulated effects of these exposures could lead to serious short- and long-term health impairments.

BINT, the most debilitating long-term consequence of a blast exposure, poses special challenges. Symptoms are not immediately visible, and may not fully develop for months/years after blast exposure. People with BINT frequently show a constellation of neurological signs, including memory loss for pre- and post-explosion events; confusion; headache; impaired sense of reality; reduced attention; memory loss for pre- and post-explosion events; confusion; impaired decision-making; and lack of social participation. Because BINT is frequently unrecognized, valuable time is often lost for preventive therapy and/or timely rehabilitation. Timely recognition of BINT is crucial to proper triage, evaluation, and monitoring of casualties for acute and chronic neurological complications. Rapid diagnosis of acute BINT and appropriate treatments of injuries, such as fulminant brain edema or severe cerebral vasospasm, could save lives immediately. Initiating rehabilitative procedures at the first symptoms of chronic BINT could prevent long-term debilitating neurological deficits. Nevertheless, despite intensified research efforts, the exact mechanisms of BINT are still not fully clarified, and their multi-factorial and multi-phase features necessitate further research.

Bearing in mind the growing number of veterans with long-term neurological deficits, we should leverage the existing knowledge on the mechanisms of blunt, civilian TBI and use the established diagnostic and treatment procedures as guidance for the development of BINT-specific diagnosis and therapy. To be able to identify similarities and differences between BINT and bTBI, a pre-requisite to achieving the highest specificity and thus successful BINT treatments, we need reliable experimental models capable of reproducing the physical properties of the injurious environment, based on the physics of blast and military-relevant injury scenarios. Using a modular, multi-chamber shock tube capable of tailoring pressure wave signatures and reproducing complex shock wave signatures seen in theater, we have developed a highly controlled, and reproducible model of blast injuries and BINT. Our well-standardized mouse BINT model mimics physiological and neurological impairments seen in servicemen exposed to blast. To reproduce a blunt impact TBI, we used a weight-drop mouse model extensively used in brain injury research. The importance of systemic, multi-organ involvement in blast injuries, thus BINT, is well demonstrated with changes in body weight where the significant weight loss remained irreversible in animals with moderate BINT. Because the mouse lifespan is approximately 45-times faster than humans, the 30-day observation period represents a significant part of their lives and relates to years in human life. Our findings indicate that while decreases in motor performance and exploratory activity, as well as stimulation of astrocytes, should be considered as generalizable consequences of a brain insult, their differing temporal profiles indicate injury-specificity that should be taken into account when diagnostic and differential diagnostic methods are developed. For example, the immediate physiological response to blast showed significant drop in arterial blood oxygen saturation, respiratory rate, and pulse distention. Such changes, previously established as blast-specific, were not seen after blunt head trauma. Moreover, motor function deficits, lack of interest in exploration (comparable to post-traumatic depression) and GFAP over-expression showed a continuing trend in animals with BINT, compared to bTBI-induced alterations that demonstrated returning tendency to pre-injury levels.

Our results emphasize the necessity of further research studies that identifies the extent of similarities and differences between BINT and blunt head injury, explain the mechanisms of those similarities and differences, and define the reliability and usefulness of diagnostic and therapeutic procedures currently in use for bTBI for treating BINT.

Apstrakt

Blast-neurotrauma (BINT) je izazvana sa kompleksom fizičkog okruženja pokrenutog eksplozijom i različitim efektima njenog udara. Kliničko iskustvo ukazuje na poseban interakciju sile udara, tela i nervnog sistema, koja izaziva složene čelijske i molekulske mehanizme koji dovode do dugotrajnih neuroloških deficit. Dokle patobiologija BINT nije potpuno rasvjetljena, rastući broj vojnih lica koja su pretrpela neurološki deficit čini neophodnim razvoj odgovarajućih i specifičnih dijagnostičkih i terapijskih procedura. U cilju identifikacije sličnosti i razlika BINT i povrede mozga izazvane tupim udarom, upotrebili smo dobro standardizovan odgovarajući model na miševima za analizu fiziolohskih (saturacija kiseonikom arterijske krvi, puls, broj udisaja i pulsni talas), funkcionalnih (motorička sposobnost, istraživačka aktivnost), i molekularnih (glalini kiseli fibrilarni protein) osećanja koja se pojavljuju do 30 dana nakon ozlade. Naši rezultati pokazuju da opšte posledice moždanog inzulta, kao što je smanjenje motoričkih sposobnosti i mentalne aktivnosti, kao i stimulacija astrocyta, imaju različit privremeni oblik, što ukazuje da specifičnost traume mora biti uzeta u razmatranje kod razvoja dijagnostičkih i diferencijalno-dijagnostičkih metoda.
REFERENCES


