Explosive blast loading neurons Explosive blast loading neurons

The effect of explosive blast loading on human neuroblastoma cells

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Abstract

Diagnosis of mild to moderate traumatic brain injury is challenging because brain tissue damage progresses slowly and is not readily detectable by conventional imaging techniques. We have developed a novel in vitro model to study primary blast loading on dissociated neurons using nitroamine explosives such as those used on the battlefield. Human neuroblastoma cells were exposed to single and triple 50-psi explosive blasts and single 100-psi blasts. Changes in membrane permeability and oxidative stress showed a significant increase for the single and triple 100-psi blast conditions compared with single 50-psi blast and controls.

Keywords: Traumatic brain injury; Explosive blast; In vitro model; Neurons; Membrane damage; Oxidative stress

Blast waves from explosive events are a major cause of traumatic brain injury (TBI) in theater. According to the Defense and Veterans Brain Injury Center, approximately 320,000 soldiers, or greater than 60% of all combat casualties, were diagnosed with a TBI between 2000 and 2014 [1]. The exact mechanisms and cause of injury (acceleration or blast overpressure), in particular injury to primary blast alone, remain largely unknown. In vitro models, which allow for the deconvolution of contributing factors to injury, are limited [2]. Recently, there have been some in vitro experimentations done with shock tubes, but due to the differences in shock wave pressure–time profiles and durations and injury severity based on animal models, the data might not reliably replicate true explosive blast conditions [3–7]. In this and our previous work, we present (to the best of our knowledge) the first-ever experimental results on the effects of realistic explosives on dissociated human neuronal cultures.

Materials and methods

Phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), antibiotic (penicillin/streptomycin, P/S), and calcein were purchased from Fisher Scientific. Fluo-4, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA), calcein-AM, and ethidium homodimer-1 were obtained from Life Technologies. Human neuroblastoma cells (SH-SY5Y) were obtained from American Type Culture Collection (ATCC) and used at a passage number of 5.

SH-SY5Y cells were plated on tissue culture-treated 24-well plates and grown for 5 days in DMEM/F12 (1:1) supplemented with 10% FBS and 5000 IU penicillin/5 mg/ml streptomycin at 1%. Just prior to the blast experiment, Hepes buffer (10 mM) was added and the well plates were sealed with sterile SealPlate covers (Excel Scientific) and then placed in a plastic bag to maintain culture sterility. Controls remained in the incubator the entire time; shams were treated identically to the experimental samples but were not subjected to blast. The experimental samples and shams were transported to the blast site and held in an oven at 37 °C until the experiments were carried out. The samples were submerged horizontally on a stage in a 10-gallon poly(methyl methacrylate) aquarium containing water heated to 37 °C. Details and pressure traces are described by Zander and coworkers [8]. The charge standoff distance of the RDX explosive (IUPAC ID of 1,3,5-trinitroperhydro-1,3,5-triazine) was adjusted to generate approximately 50- and 100-psi pressures inside the cell culture wells (stand-off distances of 365 and 315 mm, respectively). Experimental pressure readings measured 2 inches above the well plate were 49 ± 19 psi ($n = 20$) and 102 ± 21 psi ($n = 9$) for the 365- and 315-mm standoff distances, respectively. Multiple blasts were separated by 5- to 7-min intervals during which the samples were not removed from the tank. The interval used was based on the minimum amount of time required to clear the blast chamber for reentry and prepare the next explosive and does not necessarily reflect the actual time between blasts in theater. Pressure-time history and peak pressure were recorded for each blast by a pencil gauge (air) and three piezoelectric sensors (ICP model 102A, PCB Piezoelectronics, water). All analyses were conducted 24 h after the blast insult from the first 2 rows of the well plate with 6 replicates for each condition. The difference in pressure between all columns and rows 1 and 2 was not found to be significant [8]. For the live/dead assay, the cells were rinsed with PBS buffer and incubated in a 2 μ M calcein-AM and 4 μ M ethidium homodimer-1 solution for 30 min at 37 °C. To assess membrane permeability changes, the cells were washed with HBSS and incubated in 0.3 mM calcein in HBSS for 10 min at 37 °C, followed by thorough rinsing before analysis. For reactive oxygen species (ROS) evaluation, cells were rinsed with PBS and incubated in 7 μ M Carboxy-H2DCFDA for 30 min, followed by rinsing and 30 min of recovery, all at 37 °C. Samples were imaged using confocal laser scanning microscopy (CLSM) with a Zeiss LSM 700 using 488- and 543-nm lasers. Live/dead CLSM images were processed using image area analysis tools in Image J version 1.34 (National Institutes of Health), as described previously [8]. For the ROS and calcein images, imaging gains and offsets were fixed to allow semiquantitative comparison between samples. Fluorescence intensities were measured by randomly selecting individual cells using the region of interest feature and calculating the mean intensity using the histogram feature ($n = 41$ – 111 , reported in figures).

Results and discussion

One of the major contributing factors to cellular injury following blast loading is membrane damage. This damage can cause increased ion transport, such as the influx of sodium and calcium, leading to increased swelling and activation of cytoskeletal depolymerization via calpains, axonal beading, and focal adhesion disruption [9,10]. Calcein, a membrane impermanent dye, was used to assess changes in membrane permeability. Fig. 1 shows that membrane permeability was increased for cells exposed to multiple blasts at lower pressure (50 psi) as well as cells exposed to a single blast at higher pressure (100 psi). There is not a significant difference between the two conditions, which is attributed to multiple factors, including similar cumulative pressure or dose coupled with neuroprotective factors activated in the repeated blast case [3]. A single blast of 50 psi, however, was not sufficient to generate

increased membrane damage over the sham and thus was determined to be below the damage threshold. This result is in contrast to work done by others with shock tubes and our previous results with a different cell line and may be attributed to differences in the blast wave profiles and the non-adherent nature of the PC12 cell line (and thus greater shear stress experienced by the cells), respectively [3,8]. Oxidative stress can generate ROS that lead to damage of DNA, oxidation of lipids and amino acids, and ultimately cell death. Fig. 2 displays results of ROS probed with a fluorescent dye. Similar results were observed here in which the triple blast at lower pressure and the single blast at double the pressure both exhibited increased ROS generation but were not significantly different from one another. Again, a single 50-psi blast did not lead to significant ROS formation over the sham, unlike work done with a shock tube model [3]. Cell death was probed via a live/dead assay and not found to be significantly different for injured samples (single or multiple blast) compared with shams and controls ($97.7 \pm 3.9\%$ [sham] vs. $95.5 \pm 3.9\%$ [single blast 100 psi] viability). Because the cells were fixed to the well plate bottoms, we expected there to be little shear experienced by the cell. Others have found minimal cell death in excess of 200 psi in the absence of shear [11]. Although others, such as Cullen and coworkers, observed strong correlations between membrane damage and cell death, the impulse of the loading is very different due to much longer loading durations and thus not readily comparable to explosive blast loadings [12].

This work suggests that blast injury susceptibility may be dependent on cell type and that although shock tube models show similar injuries, the peak pressures at which they occur may be lower. The lower peak pressure may be a function, in part, of how the pressure is measured but may also be validating the article by Chen and Constantini for in vitro models, which claimed that injuries by shock tubes are more severe and complex than injuries from explosives [7]. Thus, more research is needed using both shock tube and explosive in vitro models to understand their differences and limitations and how best to understand and compare data.

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References

- [1] Defense and Veterans Brain Injury Center, DOD worldwide numbers for TBI, 2014. Retrieved from <http://dvbic.dcoe.mil/dod-worldwide-numbers-tbi> [2] B. Morrison, B. S. Elkin, J. Dollé, M. L. Yarmush, In vitro models of traumatic brain injury, *Annu. Rev. Biomed. Eng.* 13 (2011) 91–126.
- [3] P. Arun, J. Spadaro, J. John, R. B. Gharavi, T. B. Bentley, M. P. Nambiar, Studies on blast traumatic brain injury using in-vitro model with shock tube, *NeuroReport* 22 (2011) 379–384.
- [4] G. B. Effgen, E. W. Vogel, K. A. Lynch, A. Lobel, C. D. Hue, D. F. Meaney, C. R. Bass, B. Morrison, Isolated primary blast alters neuronal function with minimal cell death in organotypic hippocampal slice cultures, *J. Neurotrauma* 31 (2014) 1202–1210.
- [5] C. D. Hue, S. Cao, S. F. Haider, K. V. Vo, G. B. Effgen, E. Vogel, M. B. Panzer, C. R. Bass, D. F. Meaney, B. Morrison, Blood–brain barrier dysfunction after primary blast injury in vitro, *J. Neurotrauma* 30 (2013) 1652–1663.
- [6] C. D. Hue, S. Cao, C. R. Bass, D. F. Meaney, B. Morrison, Repeated primary blast injury causes delayed recovery, but not additive disruption, in an in vitro blood–brain barrier model, *J. Neurotrauma* 31 (2014) 951–960.

- [7] Y. Chen, S. Constantini, Caveats for using shock tube in blast-induced traumatic brain injury research, *Front. Neurol.* 4 (2013). doi: 10.3389/fneur.2013.00117
- [8] N. E. Zander, T. Piehler, M. Boggs, R. Banton, R. Benjamin, In vitro studies of primary explosive blast loading on neurons, *J. Neurosci. Res.* 93 (2015) 1353–1363.
- [9] D. H. Smith, D. F. Meaney, Axonal damage in traumatic brain injury, *Neuroscientist* 6 (2000) 483–495.
- [10] D. Kilinc, G. Gallo, K. A. Barbee, Mechanically-induced membrane poration causes axonal beading and localized cytoskeletal damage, *Exp. Neurol.* 212 (2008) 422–430.
- [11] R. Ravin, P. S. Blank, A. Steinkamp, S. M. Rappaport, N. Ravin, L. Bezrukov, H. Guerrero-Cazares, A. Quinones-Hinojosa, S. M. Bezrukov, J. Zimmerberg, Shear forces during blast, not abrupt changes in pressure alone, generate calcium activity in human brain cells, *PLoS One* 7(6) (2012) e39421.
- [12] D. K. Cullen, V. N. Vernekar, M. C. LaPlaca, Trauma-induced plasmalemma disruptions in three-dimensional neural cultures are dependent on strain modality and rate, *J. Neurotrauma* 28 (2011) 2219–2233.

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¹*Abbreviations used:* TBI, traumatic brain injury; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; P/S, penicillin/streptomycin; Carboxy-H2DCFDA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; CLSM, confocal laser scanning microscopy.

Fig. 1. Membrane permeability changes as a function of calcein dye uptake of SH-SY5Y neurons exposed to approximately 50- to 100-psi explosive blast: (A) fluorescence intensity calculation; (B) CLSM image of sham; (C) CLSM of single blast 50 psi; (D) CLSM of single blast 100 psi. * $P < 0.05$ compared with control, sham, 1 blast 50 psi.

Fig. 2. Reactive oxygen species changes as a function of ROS indicator uptake of SH-SY5Y neurons exposed to approximately 50- to 100-psi explosive blast: (A) fluorescence intensity calculation; (B) CLSM image of sham; (C) CLSM of single blast 50 psi; (D) CLSM of single blast 100 psi. * $P < 0.05$ compared with control, sham, 1 blast 100 psi.