Amygdala Pathology Inhibited by Steroid-loaded Hemostatic Nanoparticles after Blast Trauma

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ABSTRACT

The purpose of this study was to investigate whether hemostatic dexamethasone-loaded nanoparticles (hDNP) could reduce cellular injury and improve functional outcomes in a model of blast trauma. Blood loss is the primary cause of death at acute time points post injury in both civilian and battlefield traumas. Currently, there is a shortage in treatments for internal bleeding, especially for rapid administration in open field combat. An established polytrauma model that simulates severe injury, including primary blast lung injury and blast-induced neurotrauma (BINT), was used to evaluate hDNP. Poly(lactic-co-glycolic acid)-based nanoparticles with poly(ethylene glycol) arms were functionalized with a arginine-glycine-aspartic acid (RGD) peptide to target and adhere to activated platelets. Rats were exposed to a single "free field" blast wave at a peak overpressure of 28 psi for 2.5 ms duration using the Advanced Blast Simulator (ABS), operating above 50% lethality risk, in a side-thorax orientation (Hubbard, 2014). After injury, animals were immediately injected intravenously with hDNP, control dexamethasone-loaded nanoparticles (cDNP), or lactated ringers (LR). Sham animals were not injected or exposed to the blast wave. At one week post-blast, behavioral assays were performed preceding brain extraction for immunofluorescent staining using IBA-1 (activated microglia) and GFAP (activated astrocytes). Elevated anxiety parameters were found in the control and LR groups compared to the hDNP group. GFAP was significantly elevated in the control group compared to the hDNP and sham groups in the amygdala. Immediate intervention to assuage hemorrhage, one source for injury pathology, is crucial to mitigate debilitating injury mechanisms that lead to cognitive and emotional deficits (Shetty, 2014). It is possible that through prevention of neuroinflammatory cascades, hDNP were able to mitigate cellular injury and improve cognitive outcomes.

INTRODUCTION

Combatting internal hemorrhage within the cerebrovasculature at an early time point is crucial for limiting the amount neurologic consequences developed at a later stage (Shetty, 2014). Functionalized nanoparticles offer a wide variety of benefits and advantages compared to alternatives, such as increased biocompatibility and targeting of the injury site. Hemostatic dexamethasone-loaded nanoparticles (hDNP) attach to activated platelets and are attracted to the injury site (Bertram, 2009). In a recent U.K. study, less than fifty percent of soldiers diagnosed with primary blast lung injury (PBLI), the most common fatal blast injury, survived to reach a medical facility (Singleton, 2013). When injury occurs after blast exposure, the primary concern is to increase acute survival, but addressing mental impairment is crucial for upholding quality of life at a chronic stage. It is possible that hDNP could assist in both of these arenas, providing acute and chronic benefits. In addition to the nanoparticles offering immediate life-saving qualities, the anti-hemorrhaging qualities can result in better brain injury pathological outcomes attributed to reduced inflammatory presence as well as restoration to normoxia after initial injury.

Activated astrocytes and microglia has been shown to be upregulated in models of bTBI (Sajja, 2014; Cho, 2013; VandeVord, 2012). Cellular death is often the result of injury cascades and has been documented globally within the brain after blast injury. The injury pathway of interest in this study is glial activation and concomitant pathology. Activated glia and neuroinflammation is hypothesized to be a result of blood brain barrier (BBB) disruption. Transport of small molecules across the barrier can be costly to supporting environment of neural recovery after injury. Reb blood cells (RBCs) can infiltrate the brain during injury. Hemoglobin released from extravasated red blood cells threatens direct neuronal toxicity (Regan, 1998), which is associated with neuronal death. BBB disruption has been reported in blast studies, suggesting that it can be due to primary or secondary injury mechanisms (Yeoh, 2013). BBB breakdown are been shown to be a major factor in development of neurologic disease due to altered neurovascular dysfunction (Zlokovic, 2008). The amygdala, which is involved in fear and emotional processing, was of interest in this study due to its vulnerability to blast exposure.

Anxiety-like behavior has been reported clinically in soldiers after deployment (Matthews, 2012). Behavioral assays for anxiety-like behavior have also been utilized experimentally in animal models of blast-induced neurotrauma, with elevated plus maze, light/dark box, as well as open field assessment (Sajja, 2014; Park, 2013). This study examines potential therapeutic effects of hemostatic dexamethasone-loaded nanoparticles (hDNP) on subacute recovery in brain pathology and behavior after blast polytrauma. The ability of hDNP to mitigate injury pathways through BBB restoration should result in improved neurological function.

METHODS

Nanoparticle Synthesis and Formulation

Detailed description of nanoparticle synthesis and formulation can found in Hubbard, et al. (2015). In brief, the copolymer poly(lactic-co-glycolic acid)- poly(ε-cbz-L-lysine)-poly(ethylene glycol) (PLGA-PLL-PEG) was reacted with the peptide GRGDS in dimethyl sulfoxide (DMSO) and purified by dialysis. The steroid, dexamethasone, was dissolved in acetonitrile at a concentration of 4 mg/mL. The block copolymer, PLGA-PLL-PEG-GRGDS, was then dissolved at a concentration of 20 mg/mL in the dexamethasone (or C-6) acetonitrile solution. This solution was added dropwise to a volume of stirring PBS. Nanoparticles were then collected by coacervate precipitation. The flocculated nanoparticles were then collected by centrifugation. After rinsing, they were suspended in approximately 10 mL deionized water, snap-frozen, and lyophilized for three days. Nanoparticles were resuspended at 20 mg/mL in Lactated Ringer's solution and briefly sonicated.

Experimental Groups and Animal Procedures

The Virginia Tech Institutional Animal Care and Use Committee approved experimental protocols described herein. Prior to all experiments, male Sprague Dawley rats (~325 g, Harlan Labs, San Diego) were acclimated to a 12 hour light/dark cycle with food and water provided ad lib. Animals were exposed to a single incident pressure profile resembling a 'free-field' blast exposure at 29.2 psi (± 0.31). All animals were randomly assigned to one of four groups: Hemostatic Dexamethasone Nanoparticles (hDNP), Control Dexamethsone Nanoparticles (cDNP), Lactated Ringer's (LR), and Sham.

Prior to blast exposure, rats were anesthetized with a ketamine/xylazine solution, in accordance with the rodent weight, for sedation during blast. The shock front and blast overpressure were generated by a custom-built Advanced Blast Simulator (ABS) with an end-wave eliminator (ORA Inc. Fredericksburg, VA) located at the Center for Injury Biomechanics at Virginia Tech University. The ABS consists of a driving compression chamber attached to rectangular test section chamber with an end-wave eliminator.

A peak static overpressure was produced with compressed helium and calibrated acetate sheets (Grafix Plastics, Cleveland, OH). Pressure measurements were collected at 250 kHz using a Dash 8HF data acquisition system (Astro-Med, Inc, West Warwick, RI) and peak overpressures were calculated by determining wave speed (m/s) at the specimen position. A mesh sling was used to hold the animal during the exposure that allowed for minimal hindrance of the wave through the tube, in addition to holding the animal in a prone position with the right side of the thorax facing the shock wave driver. The animal was not allowed to impact any solid surface in order to prevent secondary injuries and this was confirmed using high-speed video (Phantom Miro eX2, Vision Research). After blast exposure, animals were immediately injected with test solution (hDNP, cDNP, or LR; 500 μL total volume) via tail vein injection. Detailed information can be found in Hubbard, et al. (2015) Sham animals underwent all procedures except for blast exposure and tail vein injection.

Behavioral Assays

At 7 seven days after blast exposure, animals underwent an open field thigmotaxis assessment. Briefly, an opaque black acrylic box with dimensions 80 x 80 x 36 cm was used for the task. Animals were acclimated in the open field box before the injury and two days after injury. The acclimation ensures that any anxiety-like traits would be due to the blast and subsequent injury progression. Activity changes were detected using EthoVision XT™ software tracking. Thigmotaxia, preference of proximity to walls, can expose fear of open, lit spaces and is displayed in animals with anxiety. Time spent along the chamber wall reflects an increased level of anxiety. Rats were videotaped for five minutes and avoidance of center square activity (i.e. anxiety-related behavior) was measured by determining the amount of time and frequency of entries into the central portion of the open field.

Tissue Processing

After seven days, animals were euthanized by transcardial perfusion of saline and 4% paraformaldehyde. Following collection, brains were stored in a 4% paraformaldehyde fixative solution. After 48 hours in fixative, the whole brains were placed in 30% sucrose solution for tissue sectioning preparation. Whole brains were embedded in Tissue-Tek® optimal cutting temperature (O.C.T.) embedding medium (Sakura Finetek USA, Inc., Torrance, CA) for cryostat processing in the coronal plane. Samples were then cut (40 µm) and sections containing amygdala nuclei were isolated (Bregma: -2.16 mm). Zeiss AxioCam ICc 1).

Immunofluorescent Staining

Immunohistochemistry was performed on amygdalar sections to evaluate levels of: glial fibrillary acidic protein (GFAP; activated astrocytes) and ionized calcium-binding adaptor molecule 1 (IBA-1; activated microglia). Samples were rinsed three times with phosphate-buffered saline (PBS) and incubated in 2% bovine serum albumin (BSA) in PBS for one hour at room temperature. Sections were then incubated with a primary antibody; anti-GFAP (1:500; Invitrogen, Carlsbad, California) or anti-IBA-1 (1:500; Biocare Medical, Concord, California) overnight at 4ºC. Both primary antibodies were only labeled separately on different amygdalar sections. After a PBS wash, the samples were incubated for 1.5 hours with Alexa Fluor 555 anti-rabbit IgG antibody, or FITC anti-rat IgG antibody (Invitrogen, Carlsbad, California). After three PBS washes (five minutes each), samples were mounted, air dried and coverslipped with prolong antifade gold reagent with 6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Sections were examined under Zeiss fluorescence microscope at 20X magnification under appropriate fluorescent filters and images were taken by Zeiss AxioCam ICc 1. For all images, quantification (ImageJ software; NIH, Bethesda, MD) was based on fluorescence intensity after thresholding to eliminate background color.

Flurojade-B Staining

Sections of the amygdala were stained with Fluorojade-B (FJB) to identify degenerating neurons. Tissue sections were incubated in the solution of 1% alkaline (NaOH) in 80% ethanol,

and then hydrated in 70% ethanol and distilled water. The sections were then incubated in a solution of 0.06% potassium permanganate, rinsed in distilled water, and incubated in a 0.0004% solution of FJB (Histo-chem Inc., Jefferson, AR). Sections were then rinsed in distilled water, air-dried, and placed on slide warmer until fully dry. The dry slides were cleared in xylene and mounted with DPX (Sigma-Aldrich Co. Ltd, St. Louis, MO). Sections were examined at 20x on a Zeiss microscope, and analysis was conducted. The green fluorescent intensity of each image of amygdalar nuclei was quantified after thresholding to eliminate background color.

Statistical Analysis

Statistical differences between the treatment groups were assessed with analysis of variance (ANOVA) using LSD post-hoc test. All statistical analyzes were performed using JMP Pro 10 (SAS Institute, Cary, NC) and $p < 0.05$ considered statistically significant. Unless indicated otherwise, data are presented as mean ± standard error of the mean, or SEM.

RESULTS

Weight assessment at seven days post-blast has shown subacute recovery by the hDNP group, displayed by significant difference from the cDNP group (Figure 1). The data is compared to baseline (weight at time of injury), in which none of the groups were significantly different. Two days after blast there were no difference between hDNP and cDNP groups.

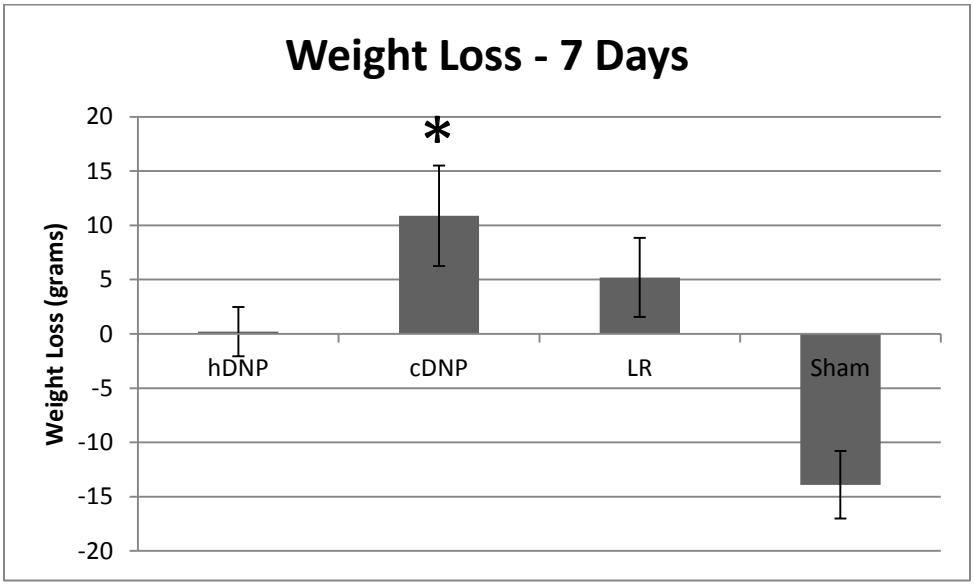

Figure 1: The hDNP and sham groups were significantly different from the cDNP group (* - $p<0.05$) at seven days after blast.

The hDNP group displayed significantly different anxiety behavior compared to LR at seven days post-blast (Figures 2 and 3). The hDNP group displays recovery by seven days and there was no difference compared to sham. Prevalence for the walls (sign of anxiety) is seen more in the cDNP and LR groups in animal tracking over five minutes.

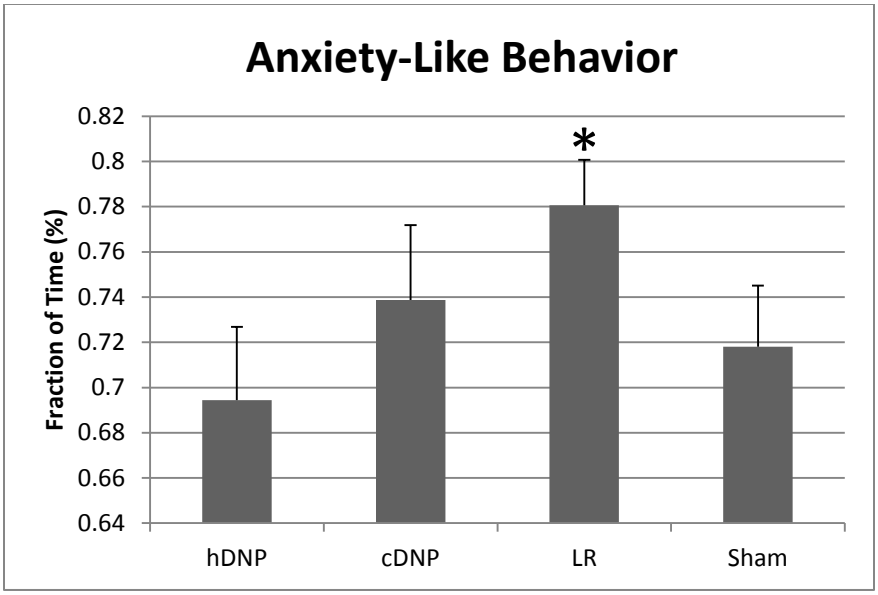

Figure 2: The hDNP was significantly different from the LR group (* - $p<0.05$) at seven days after blast.

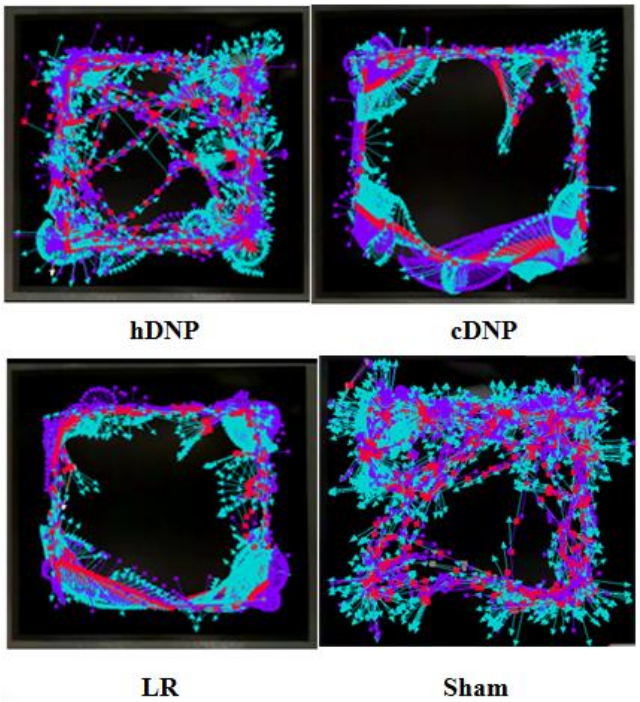

Figure 3: Image depicts a representative tracking of thigmotaxis in the treatment groups. Blue arrows signify the head point of the animal, red squares signify the body position of the animal, and purple squares signify the tail position of the animal.

For microglial expression and activation, the hDNP group was not statistically significant but was trending away from the LR group (^ - $p<0.13$) at seven days after blast (Figures 4 and 5). Amygdalar images show increased activation in the cDNP and LR groups.

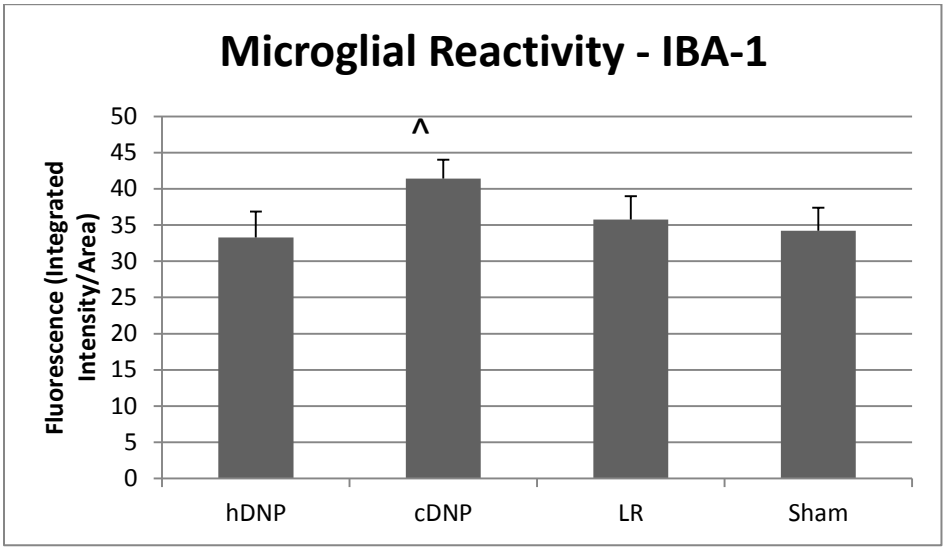

Figure 4: Integrated density is normalized to area of image according to the amount of red fluorescence representing IBA-1 expression in the amygdala. ^ $p < 0.13$

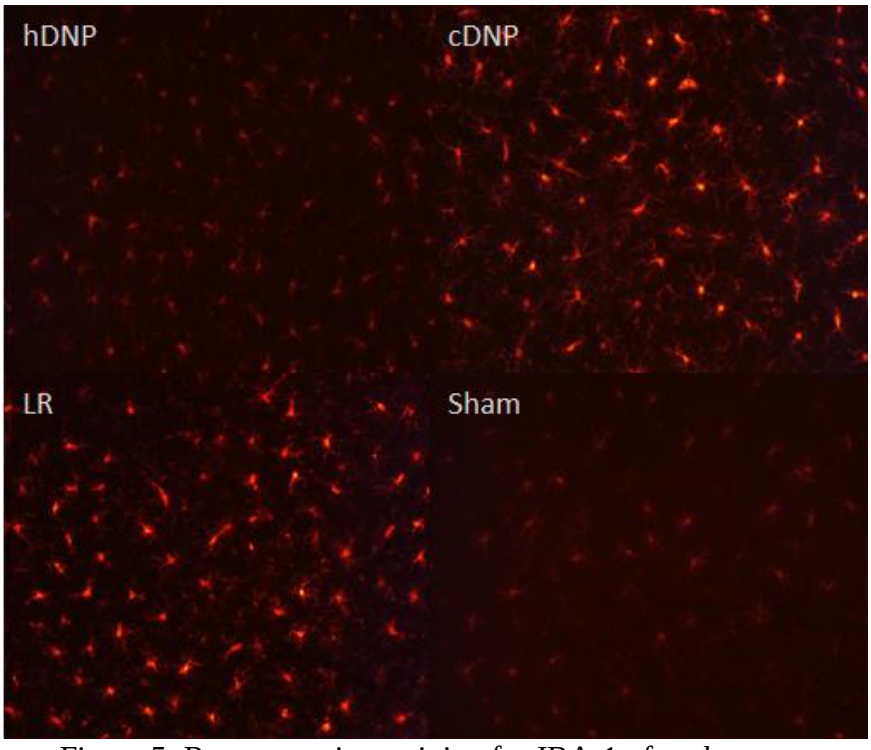

Figure 5: Representative staining for IBA-1 of each group.

For astrocyte activation, the cDNP group was significantly different from the hDNP and sham groups (Figures 6 and 7). The LR group was elevated though not significant.

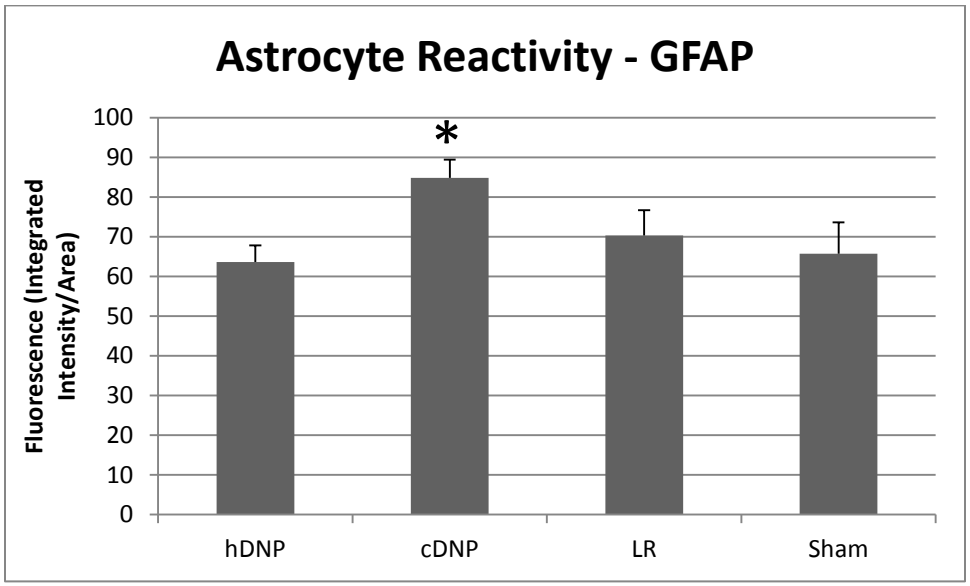

Figure 6. Integrated density is normalized to area of image according to the amount of green fluorescence representing GFAP expression in the amygdala. * - p-value < 0.05.

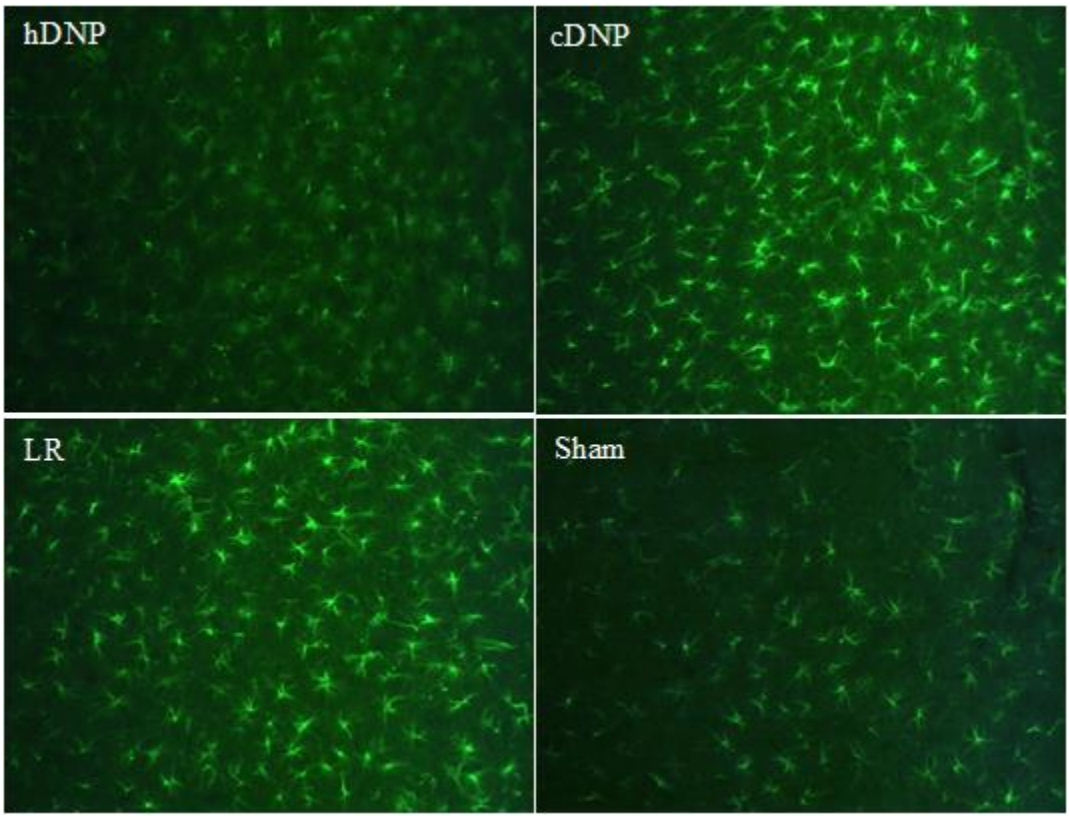

Figure 7: Representative staining for GFAP of each group.

The cDNP and LR groups were significantly different from the hDNP and sham groups (Figure 8) for neurodegeneration in the amygdala.

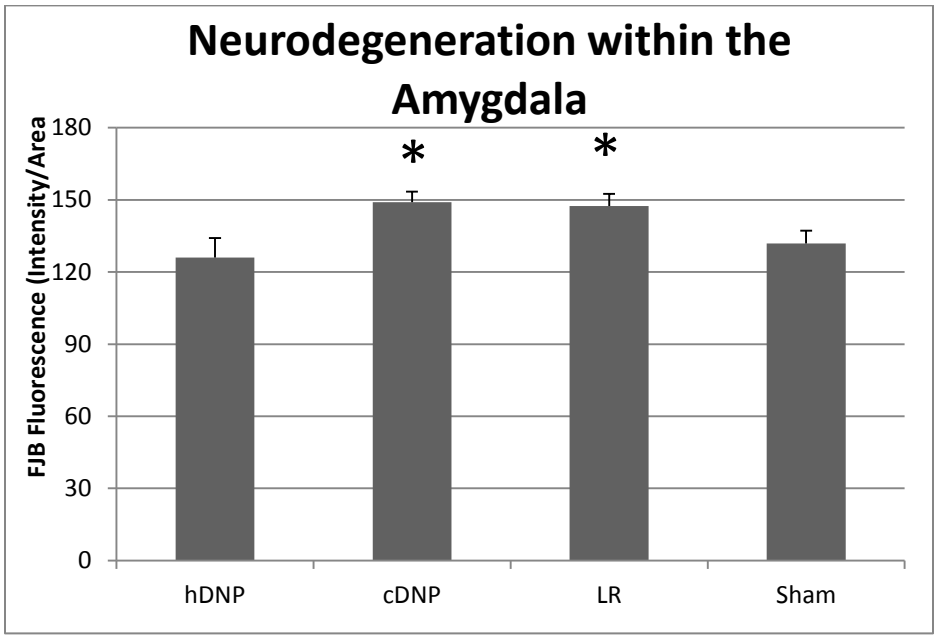

Figure 8. Integrated Density was normalized to area of image according to the amount of fluorescence representing FJB expression in the amygdala. * p-value < 0.05

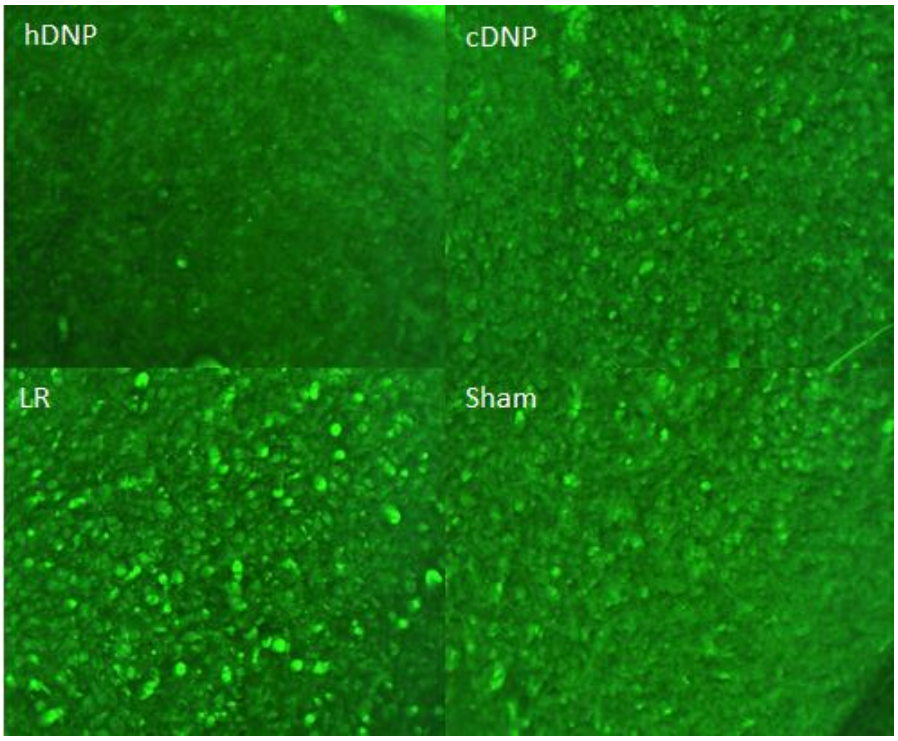

Figure 9: Representative staining for FJB of each group.

DISCUSSION

Weight loss has been documented to occur after bTBI injury over 27 psi exposure acutely up to five days (Skotak, 2013; Wang, 2011). Since weight loss has been shown to be associated with higher blast exposure, weight loss is an indicator of injury in our blast model. Food intake has been shown to decrease in rats exposed to blast overpressure (BOP), possibly due to taste/olfaction changes (Bauman, 1997). Increased weight in the hDNP compared to control blast injury groups demonstrates systemic recovery.

The presence of anxiety-like behavior has been reported in BINT literature. In a repeated blast exposure model, it is shown that repeated stressors compiled with repeated blast can exacerbate anxiety levels immediately after exposure (Kamnaksh, 2011). Anxiety has also been reported 7 days after mild blast exposure in Sajja, et al (2014). A study examining the relation of blast exposure to post-traumatic syndrome disorder (PTSD) in a rat model found increased anxiety parameters from repeated mild exposure, in the absence of psychological stressors (Elder, 2012). Chronic levels of increased stathmin-1 were reported at eight months after repeated mild blast exposure, which is responsible for fear responses (Elder, 2012). In a rat model of mild bTBI, minocycline was administered and negated anxiety seen in injured animals at 8 days post-injury (Kovesdi, 2012). The reduction in thigmotaxia, representative of anxiety, in the hDNP group is a promising result for the therapeutic to have a lasting impact on neurological recovery.

The amygdala has been studied in blast-induced TBI studies and has relation to anxiety-like behavior. In a murine blast TBI model, anxiety was seen in open field activity in 25-40 psi blast exposures at one week following blast, in addition to neuronal reduction and anxiety in the 50-60 psi exposure group (Heldt, 2014). Also, protein levels of neuronal and glial damage were seen increased in amygdala (Kovesdi, 2012). Pressure dependence can also play a role in display of neurologic impairment and elevated glial activity and neurodegeneration (VandeVord, 2012). A severe exposure, 28 psi, could produce extensive amygdalar injury that has not been seen due to anatomical positioning of the amygdala. Five days after initiation of injury, astrocyte levels were increased in amygdala in repeated mild TBI rat model along with increased levels of VEGF, which can be upregulated by hypoxic conditions and can regulate vascular permeability (Kamnaksh, 2011). Elevated levels of activated microglia and astrocytes have also been reported at 7 days post-blast in the amygdala (Sajja, 2014). In another mild TBI injury model by Perez Polo, et al. (2015), increased microglia activation was seen in the amygdala at 6 hours and 30 days after injury and BBB impairment in the hippocampus at 6 hours after injury. Recovery of the BBB was noticed at 30 days after blast (Perez-Polo, 2015). Recovery is likely not stagnant with varying levels of blast exposure and as long as there is compromise, there is potential for debilitating neurological consequence. This result furthers the pressure dependence hypothesis, with severe exposure stimulating an on-going presence of injury pathology. Activated glia found in the amygdala demonstrates on-going injury cascades at seven days in the current study. Significantly increased glial activation, as well as apoptosis, in the cDNP group shows that inflammatory injury cascades are still at play subacutely. With the benefit of injury site targeting and hemostasis, these injury mechanisms were not observed in the hDNP group.

Matthews, et al. (2012) reported that veterans suffering from major depressive disorder (MDD) due to blast exposure displayed amygdalar hyperactivity. The amygdala is crucial in processing fear and negative emotions and can be hyper-regulated after blast-related TBI, confirmed by clinical imaging data (Matthews, 2012). Increased MEG activity is seen in the amygdala for individuals that have developed PTSD and hyperactivity has been reported in the amygdala. (Huang, 2015) Altered neural connectivity, possibly mediated by glial activation and recovery mechanisms after injury, can produce negative emotional patterns that can affect everyday life. PTSD is often accompanied by symptoms such as anxiety. Prevalence of clinician-rated PTSD symptoms was higher in veterans with a history of TBI (Davis, 2013).

BBB disruption has been seen in various models, such as epilepsy and Alzheimier's disease, and over time, can culminate into neuronal dysfuction (Obermeier, 2013; Sheen, 2011; Altman, 2010; Deane, 2007). Astroglial abnormalities have been associated with BBB breakdown, occasionally resulting from dystrophin reduction (cortex) (Sheen, 2011). It has been shown that there can be BBB disruption and increased permeability due to hypoxia, which occurs acutely in this full-body polytrauma model (Kaur, 2008).

The mechanics behind amygdala position within the brain is poorly understood but its anatomical location could make it more susceptible to severe exposure. Shearing of vessels can cause microhemorrhaging. While some studies support the mechanics of brain injury in blast stemming from a vascular surge, we hypothesize that skull flexure gives way to a deformability of the viscoelastic brain that induces injury internally (Gean, 2014). The neuroanatomical position of the amygdala inside the rat brain would make it susceptible to flexure of the ventral side of the skull due to high static overpressure. The mechanism in which the blast wave interacts with different tissues in the brain is not fully understood. One hypothesis mentioned in Kuehn, et al. (2011) is that the exhaustion of energy at density boundaries can cause injury in tissue. Similar to the air-water interface that is found in the lung, a vulnerable organ in BOP exposure, the water-lipid interface occurring where the CSF touches the pial surface can also form a density gradient. While mortality from direct blast exposure to the brain is not seen until 500 kPa peak overpressure, it can be assumed that microcontusion and microhemorrhaging of the BBB can occur starting at 200 kPa peak overpressure. (Kuehn, 2011) Micro-lesions in the BBB were seen in side blast exposure as low as 230 kPa. (Yeoh, 2013) The vascular injury seen in the brain gives blast-induced neurotrauma a unique identity that requires different therapeutics compared to traditional TBI. (Yeoh, 2013)

The therapeutic effect of dexamethasone has been previously looked at in other injury models (Hubbard, 2015; Araz, 2013; Lee, 2015). Dexamethasone has been shown to reduce inflammation due to amyloid beta in the cerebrovasculature, in relation to AD (Previti, 2006). Hemostatic nanoparticles have been used in treatment of acute hemorrhaging in injury models (Bertram, 2009; Shoffstall, 2012; Lashof-Sullivan, 2014). Since the cDNP group had less injury recovery and was comparable to the LR-inject blast group, we can place emphasis on the need for hemostasis at the injury site with the targeting ability of the nanoparticles. A recent review of BBB and its repair has described the only applicable BBB therapeutic as glucocorticosteroid (GC) treatment (Obermeier, 2013). Dexamethasone, an anti-inflammatory GC, has been shown to inhibit matrix metalloproteinase (MMP) levels and consequently improve vessel wall integrity by preserving BBB components (Forster, 2007).

CONCLUSIONS

Neuroprotective drugs capable of halting or mitigating secondary changes following blast (shock wave induced inflammation and/or BBB disruption) may considerably ease or slow down the development of subsequent neurological and neuropsychiatric impairments such as cognitive problems, non-specific mental and emotional symptoms, and PTSD (Shetty, 2014). hDNP are a potential therapeutic for administration in a clinical or combat setting. Translation would provide a viable option for mitigation of internal bleeding, which contributes to alleviation of on-going injury mechanisms. The prevention of subacute and chronic inflammatory pathways and BBB dysfunction can improve neurological function. Future studies will evaluate the effect on oxidative and hypoxia-related proteins after hDNP administration post-trauma.

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