Research Article

Intracranial venous injury, thrombosis and repair as hallmarks of mild blast traumatic brain injury in rats: Lessons from histological and immunohistochemical studies of decalcified sectioned heads and correlative microarray analysis

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ABSTRACT

Background. Many previous experimental studies of blast wave effects have reported vascular and parenchymal injury in brains extracted from the skulls prior to histopathological assessment. Brain removal disrupts vasculature and structural features of the meninges that may be sources of signs and symptoms of mild traumatic brain injury, particular at lower blast overpressures (<5 psi peak).

New Method. Immunohistochemical and histopathological studies have been conducted in sections from decalcified, paraffin embedded, histologically sectioned whole rat heads. These sections preserve the entire cranial contents in situ, and permit evaluation of the inner ear, central nervous system and associated vasculature. The findings could also be correlated with mRNA expression patterns from whole brains subjected to similar treatment.

Results. Lower levels of blast wave exposure produce primarily vascular effects in rats. Messenger RNA profiles of the whole brains showed evidence of both blast intensity and time dependent effects on vascular wound healing markers. The rats exposed to 10-11 psi overpressure tended to show a similar pattern of mRNA expression changes in these vascular repair and inflammatory pathways as rats exposed to approximately 5 psi overpressure, but the changes were greater. The changes in mRNA expression after a 14-15 psi exposure were different and suggestive of more severe injury, particularly for DNA repair, lymphocyte activation and lymphocyte migration pathways. Histopathological examination of decalcified heads revealed that even 2.5-7.9 psi blast exposures produced a high prevalence of mild venous hemorrhage and thrombosis (accompanied by inflammatory markers) in the inner ear, vertebrobasilar circulation, hippocampal choroidal fissure and the veins associated with velum interpositum.

Comparison with Existing Method(s). The sites of vascular injury would not have been included in specimens extracted from the skull prior to processing.

Conclusions. The isolated regions of intravascular coagulation in small veins and the isolated, very small venous hemorrhages in the subarachnoid space are worthy of consideration as factors in both healing and chronic sequelae of mild blast concussion. Although small, remnants persisted in the subarachnoid space even 42 days after a single blast exposure. The high prevalence of very mild subdural and subarachnoid hemorrhage may be a target for clinical management.

1. Introduction

Blast related mild traumatic brain injury (mTBI) has been called a signature injury of modern warfare. Self-reports suggest that as many as 19.5% of military personnel deployed in Southwest Asia sustained a TBI [1]. The Defense Centers of Excellence for Psychological Health and Traumatic Brain Injury (DCOE) estimates that over 30,000 military members last year were diagnosed with a new TBI [2]. Accidental explosions and terrorist improvised explosive devices also create mTBI casualties among civilians. Long-term effects of mTBI include pathological changes closely related to neurodegenerative disease, postconcussive syndrome, and long-term inflammatory changes throughout the brain [3, 4].

Although radiological findings are generally subclinical in mild TBI, there is increasing interest in more subtle vascular findings such as dilation of perivascular spaces [5], fractional anisotropy changes and vascular changes that can be detected with susceptibility-weighted imaging [6]. Subdural hematoma, cerebral contusion, and subarachnoid hematoma are among the pathological features associated classically with TBI [7]. Effects may be acute or delayed; for example, subdural hematoma can emerge in acute, subacute or chronic stages after injury [7]. Because in the brain (like lung, liver, kidney, heart and GI tract) the intracranial vascular contents are soft tissues, they are susceptible to similar blast injury effects, which include local plasma extravasation (local edema), local hemorrhage (including perivascular ring hemorrhage), disseminated intravascular coagulation (a term that includes microthrombi in small vessels) and introduction of air emboli [8]. Small injuries that are isolated or distributed diffusely could easily elude clinical detection with imaging protocols. In addition, localized hemorrhage is difficult to detect with imaging in extensions of the subdural space such as the velum interpositum and transverse cerebral fissure. However, these types of injuries can be detected in histological sections of decalcified whole heads of experimental animals.

Chemical decalcification of bony tissues prior to embedding and sectioning has long been used for histological studies of the structure of soft tissues encased in teeth and bone [9, 10]. In the neurosciences, decalcification has been used frequently in studies of inner ear structures within the temporal bone, including neural and non-neural tissues. For example, studies of paraffin or celloidin embedded, decalcified human temporal bones from autopsy have examined normal and pathologic structure with standard histological stains and immunohistochemistry for neuronal and inflammatory markers [11-16]. Because these preparatory methods allow histological analysis in situ of fragile tissues such as intracerebral veins and meninges, it seems logical to section whole decalcified rodent heads for analysis of the effects of blast trauma to the intracranial contents.

This study assessed the intracranial injury in rats after shock tube delivery of single, low level Friedlander waves (2.9 psi [20 kPa] - 17.5 psi [120.7 kPa] peak overpressure). The histological analyses spanned acute (2 hour post-exposure) to chronic (42 days post-exposure); changes in whole brain mRNA expression were assessed in separate groups at only the shorter survival times (2 – 72 hours). The analyses revealed previously undescribed vascular injury patterns in the inner ear and within the subarachnoid space and evidence of small subarchnoid hemorrhages and thrombosis in small veins on the brain surface and subarachnoid space.

2. METHODS

2.1 Blast Overpressure Delivery

A 30" cast aluminum tube (10" inner and 8" outer diameter) compression chamber was used to generate the blast pressure waves. The tube houses an adjustable reservoir chamber that releases a compressed air volume ranging from 1570 to 7850 cm³ through a PVP film diaphragm. A second 15-inch tubular portion attaches to the wave generation chamber and serves as the animal exposure and holding area (Figure 0, left panel). A 1.6 hp compressor (Sears Inc) produces up to 175 psi in the

compression chamber. Between the reservoir chamber and the animal holding area is a variable aperture that holds the PVP film diaphragm. Three, four or five-inch diameter apertures can be interchangeably placed in this area to adjust the wave intensity from a mild, moderate or more severe BOP exposures. The input to peak output pressures are linear over the range of 0 – 100 psi, and the velocity of the BOP wave was measured at 1221 feet/sec at six inches from the compression chamber aperture. The exposure peak intensity levels for this study ranged from 4.7-20 psi.

Adult female Sprague-Dawley rats were anesthetized intramuscularly with a ketamine (80 mg/kg)-xylazine (5 mg/kg) cocktail, placed horizontally in the holding/exposure area and tethered to a removable wire rack either six or 12 inches from the film diaphragm. The head faced the blast source. The blast overpressure (BOP) wave is measured with Tucker Davis Technologies (TDT) System 3 and Endevco pressure transducers (model 8510B-200) rated for 200 PSI and electronic conditioners interfaced with a computer. Figure 1 shows examples of overpressure/underpressure Friedlander waves generated by this system. Brain and lung tissues were harvested at 2 hours, 24 hours and 72 hours after single BOP exposure. Behavioral assessments were conducted daily for Roto-rod performance and at least one week post-BOP for open field activity.

****Figure 1 About Here**

All animals were monitored for respiratory rate, heart rate and blood oxygen saturation using a SurgiVet Pulse oximeter prior to, during the exposure and for the entire recovery period after the exposure. Once functional and cognitive measures are complete on surviving blast-exposed animals, animals were euthanized for collection of tissues, body fluids and blood.

2.2 Tissue preparation

Brain tissues from one set of rats were harvested at NMCS D at 2, 24 and 72 hours after blast exposure, frozen on dry ice, and shipped to University of Pittsburgh for RT-PCR analysis. The tissues were homogenized and the mRNA was extracted by standard methods with commercially available reagents (Quiagen RNeasy lysis reagent, RNeasy kits). RT-PCR microarray analyses with standard commercially available products and qRT-PCR on individual mRNAs were used to examine changes in specific response pathways (Applied Biosystems 7300 Real Time PCR System). The arrays include SuperArray Biosciences RT² Profiler™ PCR Arrays PARN-024A (rat angiogenesis microarray), PARN-003A (rat stress and toxicity pathway microarray), PARN-060A (rat neurotransmitter array) and PARN-011A (rat inflammatory cytokine/chemokine and receptor array) for assays on each blast overpressure exposure group. The microarrays were performed in at least duplicate on pooled samples of equal amounts of cDNA from 3-5 animals in each exposure or control group, to yield an estimate of the mean mRNA response patterns. The means of the cycle threshold (C_t) values for mRNAs for ribosomal protein large P1 (Rplp1), lactic acid dehydrogenase A (Ldha), beta actin (Actb), hypoxanthine guanine phosphoribosyltransferase (Hprt) and ribosomal protein L13A (Rpl13a) was used for housekeeping gene normalization; it was subtracted from each C_t value to obtain ΔC_t . The difference between the average ΔC_t in each shock wave intensity group and the average ΔC_t in the control group yielded the cycle threshold change, $\Delta\Delta C_t$. The fold change in mRNA expression is reported as $2^{-\Delta\Delta C_t}$. Data were expressed as $-\Delta\Delta C_t$ for statistical analyses.

After survival times ranging from 2 hours to 42 days, sham and blast exposed rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.1M PBS, followed by paraformaldehyde-lysine-periodate fixative. The intact heads were post-fixed in 4% paraformaldehyde for 24 h at RT, decalcified in 10% formic acid to chemical testing criterion and neutralized in overnight 5% sodium sulfate. The heads were trimmed with a horizontal cut at the ventral edge of the bullae and a transverse cut at the rostral margin of the orbit, and then embedded in paraffin.

Sections were cut at 8-10 microns in the horizontal plane. Every fiftieth section was stained with hematoxylin and eosin for standard histopathological analysis. For immunohistochemistry, sections are incubated sequentially in the following solutions at RT: 5% normal donkey serum (NDS) in 0.1M PBS for 2h; primary antisera in 0.1M PBS for 72 hr and 0.1M PBS for 15 min. After processing with secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA: anti-goat IgG: diluted 1:500) in 0.1M PBS for 24 h; 0.1M PBS for 15 min), the tissue was processed with a standard ABC-peroxidase method (Vectastain™) and a DAB chromagen. For controls, primary antibodies were either pre-absorbed with each control peptide (1:50) or the primary antibody was omitted.

3. RESULTS

3.1 Microarray Findings. There were both blast intensity and time dependent changes in mRNA expression. Quantitative PCR microarray analysis showed different temporal patterns of expression of wound-related mRNAs as a function of the intensity of single shock wave exposures (Figures 2-5). The data are displayed as differences in PCR cycle threshold relative to control ($-\Delta\Delta C_t$), which represent the base 2 logarithm of the differences in copy number. We will consider only a doubling ($-\Delta\Delta C_t=1$) as a threshold for describing up-regulation and a halving ($-\Delta\Delta C_t=-1$) for down-regulation.

Wound healing with vascular injury involves an intricate interplay between inflammatory processes, matrix reorganization, oxidative stress, degenerative processes and regenerative mechanisms that include angiogenesis and angiostasis mechanisms.

****Figure 2 ABOUT HERE**

****Figure 3 About Here**

3.1.1. Vascular and Matrix Remodeling Substrates. Expression of groups of mRNAs associated with angiogenesis (Angpt1, Ctgf, Ecgf1, Ereg, Nrp1, Ptgs1, Tek and Vegfa), angiostasis / angiogenesis inhibition (Angpt1, Bai1, Col18a1, Flt1 and Vegfb), associated fibroblast proliferation (Akt1, Fgf1, Fgf2, Fgfr3, Fn1, Ifnb1, Pdgfa and Pdgfb) and integrins (Itga5, Itgam, Itgav, Itgb2 and Itgb3) varied with both blast intensity and time after the blast (Figures 2-5). In the 5 psi peak overpressure group, there was an upregulation of brain angiogenesis inhibitor 1 (Bai1) at 2 hours that persisted through 72 hours, accompanied by a delayed upregulation in the endostatin precursor Col18A1 at 24 and 72 hours. The angiostasis and angiogenesis inhibition associated vascular endothelial growth factor B (Vegfb) was down-regulated transiently at 2 hours after 5 PSI blast exposure, and the mRNA for the local proliferative molecule Ereg was down-regulated significantly and progressively between 2 and 72 hours after exposure. The changes in mRNA for the fibroblast marker battery and the integrins did not reach the two-fold criterion for the 5 psi group.

A different pattern appeared in the 10-11 psi and 14-15 psi shock wave exposure groups. Among mRNAs related to angiogenesis and fibronectin/integrin pathways, the magnitude of time-related up-regulation of Angpt1, Ctgf, Ecgf1, Ereg, Nrp1, Tek, Vegfa, Itga5, Fn1, Itgam, Itgav, Itgb2 and Itgb3 was greater in the higher magnitude exposure group, as was the down-regulation of Ptgs1 at 72 hours after either exposure. A similar pattern appeared for a sample of mRNAs for angiostatic/angiogenesis inhibition factors and the fibronectin/integrin battery. Elevated mRNA copy numbers ($-\Delta\Delta C_t \geq 1$) for Angpt1, Bai1, Col18a1 and Flt1 were present at 2, 24 and 72 hours, and were greater in the 14-15 psi than the 10-11 psi group. Vegfb mRNA was elevated ($-\Delta\Delta C_t \geq 1$) only at the higher blast exposure. The sampled fibroblast markers also showed an enhanced upregulation in the 14-15 psi than the 10-11 psi exposure group. For the 10-11 psi exposure, Fgf2, Fgfr3, Fn1, and Ifnb1 mRNA copy numbers were elevated ($-\Delta\Delta C_t \geq 1$) at all three times, joined by elevated ($-\Delta\Delta C_t \geq 1$) Akt1 and Fgf1 findings at 24 hours after the shock wave. By contrast, Akt1, Fgf1, Fgf2, Fgfr3, Fn1, Ifnb1, and Pdgfb mRNAs were

all elevated ($-\Delta\Delta C_t \geq 1$) at all times after the 14-15 psi shock wave exposure, with Pdgfa elevated only at 2 and 24 hour time points. These findings suggest collectively that vascular remodeling activity was increased with shock wave intensity, and that it is even detectable for the lowest (5 psi) exposure.

Two mRNAs for the major basement membrane components laminin $\alpha 5$ (Lama5) subunit and collagen IV $\alpha 3$ (Col4a3) subunit increased expression with the intensity of the shock wave. However, changes in mRNAs related to matrix metalloproteinase (MMP) turnover suggest that there are different patterns of matrix repair in the low and higher shock wave exposures. For the 5 psi exposure group, mRNA changes suggested concerted collagen IV remodeling activity with no or decreased Lama5 transcriptional activity. There was a massive and persistent up-regulation of mRNA for Mmp9, which degrades type IV and V collagens, accompanied by a modest up-regulation of Col4a3 mRNA, a down-regulation of the Mmp9-associated inhibitor Timp2 mRNA and a down-regulation ($-\Delta\Delta C_t \geq -1$ at 72 hours) of Lama5 mRNA. For the higher magnitude shock wave groups, there was a concurrent up-regulation ($-\Delta\Delta C_t \geq 1$ of Mmp2, Mmp19, Timp2 and Timp3 mRNA at 24 hours in 10-11 psi group and at 2, 24 and 72 hours in the 14-15 psi exposure group. It is interesting to note that the concurrent down-regulation of Mmp9 and up-regulation of Timp2 mRNA at 72 hours after the 14-15 psi exposure is opposite to the response in the 5 psi group.

***Figure 4 about here

3.1.2. Lymphocyte Activation and Migration Substrates. Markers for lymphocyte activation and migration suggest that there is a qualitative difference in the responses to 14-15 psi shock wave versus lower than 11 psi shock wave exposures. The responses appeared similar and scalable at 5 psi and 10-11 psi exposures. The mRNA for a marker of T and B cell trafficking in veinules (Ccr7) increased ($-\Delta\Delta C_t \geq 1$) within 2 hours after 5 psi exposure and remained elevated at both 24 and 72 hours, without a change in

mRNA expression for its ligands Ccl19 and Ccl21b (Fig. 6). This effect was accompanied 2 hours after shock exposure by a transient increase ($-\Delta\Delta C_t \geq 1$) in mRNA for Bir1/Cxcr5a, which is associated with B cell migration and a decrease ($-\Delta\Delta C_t \leq -1$) in mRNA for Cx3cl1, which is a factor promoting lymphocyte adhesion to veinules. Finally, there was a transient increase in interleukin 10 mRNA at 24 hours after exposure. These effects on substrates for lymphocyte migration were augmented in duration and magnitude in the group exposed to a 10-11 psi shock wave. The increases in Ccr7 were accompanied by sustained up-regulation of mRNA for its ligand Ccl21b (2-72 hours) and a transient up-regulation of a second ligand Ccl19 mRNA at 24 hours post-exposure. There was also an earlier, greater and sustained up-regulation of Bir1/Cxcr5a mRNA, accompanied by a larger and longer duration down-regulation of Cx3cl1. A stronger inflammatory component to the response is also suggested by the augmented changes in Il10 mRNA at 2 and 24 hours after exposure, coupled with up-regulation of both lymphotoxin alpha mRNA at 24 hours and lymphotoxin beta mRNA at 24 hours after exposure.

The response pattern differed profoundly for the 14-15 psi exposure group. First, within 2 hours after exposure, the significant changes in Bir1/Cxcr5a, Ccr7 and its ligands (Ccl19 and Ccl21b), Cx3cl1 and Il10 were opposite to the polarity of responses at 10-11 psi. Second, there was only a transient upregulation (at 24 hours post-exposure) of mRNAs related to lymphocyte migration: Bir1/Cxcr5a, Ccr7 and Ccl21b. Third, there was a persistent upregulation of lymphotoxin alpha mRNA within two hours after exposure; the down-regulation of mRNA for Il10 (which counters lymphotoxin alpha effects) at 2 hours, followed by a transient upregulation at 24 hours suggests polyphasic effects on CD8 lymphocytes. Finally, the 72 hour response resembled the 2 hour response, including the up-regulation of Cx3cl1 mRNA.

The mRNAs for CXC motif chemokine receptor 3 and ligands, on the other hand, showed little change after 5 psi exposures, but a very marked response in the 10-11 psi exposed animals.

Paradoxically, the receptor mRNA upregulation was observed at higher blast exposures, but with a

down-regulation of ligand mRNAs. Immunohistochemical results suggest that the receptor mRNA up-regulation is related to an up-regulation of the protein in cortical white matter astrocytes (Figure).

**Figure 5 ABOUT HERE

3.1.3. Oxidative stress and DNA repair. The mRNA expression associated with a panel of regulators of cellular free radical levels (Fmo1, Fmo4, Fmo5, Gpx1, Gpx2, Gsr, Gstm1, Gstm2, Sod1 and Sod2) showed no change in the 5 PSI exposure group. However, there was a slight increase in mRNA for cyclin G1 (Ccng1) at all survival times and an increase in the dual incision nucleotide excision repair enzyme Ercc1 at 2 hours, suggestive of reparable DNA damage.

The 10-11 psi exposure group showed a much greater response. The shock wave elicited an up-regulation of free radical-related mRNAs for flavin monooxygenase 1 (Fmo1), glutathione peroxidase 2 (Gspx1) and the cytoplasmic superoxide dismutase 1 (Sod1) within 2 hours, accompanied by a strong down-regulation of glutathione S-transferase mu 1 (Gstm1). Despite an increase in cyclin G1 mRNA expression, there was no significant early change in mRNAs related to DNA repair. Within 24 hours, the free radical metabolism-related reaction expanded into a modest up-regulation ($-\Delta\Delta C_t$ between 1 and 2) that included Fmo1, Fmo5, Gpx2, Gsr, Gsr, Gstm2, Sod1 and Sod2. It was accompanied by up-regulation mRNA for a mediator of dual incision nucleotide excision repair (ERCC4-like sequence) and down-regulation of Xrcc4 mRNA, which encodes an enzyme for DNA double stranded break repair. This concurrent free radical and DNA repair response coincided with a transient increase in mRNA for two major enzymes for heme catabolism to biliverdin, heme oxidase 1 (Hmox1, $-\Delta\Delta C_t=1.02$) and heme oxidase (Hmox2, $-\Delta\Delta C_t=0.89$), as well as mRNA for Chk1, an enzyme that arrests the cell cycle in G1 for DNA repair. The response waned by 72 hours to sustained increases in only Fmo1 and Sod1, and a marked decrease in Gstm2 mRNA, accompanied by a down-regulation of the DNA repair mRNA battery.

The responses differed considerably after a 14-15 psi shock wave exposure. There was a prominent DNA repair-related up-regulatory response, which presented at 2 and 24 hours with up-

regulation of mRNA for Tp53 (Figure 5), dual incision nucleotide excision repair (ERCC4-like sequence), nucleotide excision repair (Rad23a), base excision repair (Ung and Xrcc1) and double stranded break repair (XRCC2-like, Rad50 and Xrcc4) enzymes. These responses were joined at 72 hours by up-regulation of mRNA for another dual incision nucleotide excision repair enzyme (Ercc1) and a major up-regulation of a transcription coupled NER enzyme (Ercc2), as well as augmented mRNA for cyclin G1 (Ccng1), Chek2, Ccnd1, and Growth arrest and DNA-damage-inducible protein alpha (GADD45a). The free radical metabolism-related mRNAs, by contrast, were relatively unaffected before 72 hours, showing a sustained up-regulation of Sod1 and Sod2, a down-regulation of Gsr, and up-regulation of Fmo1 at 24 hours. At 72 hours, though, there was a strong and concerted up-regulation ($-\Delta\Delta C_t \geq 1$) of all ten mRNAs in this screening battery. As observed at 24 hours in the 10-11 psi exposure group, this higher magnitude, concurrent free radical response, DNA repair response and growth arrest at the G1/S checkpoint response coincided with augmented up-regulation of mRNA for both Hmox1 ($-\Delta\Delta C_t = 3.56$) and Hmox2 ($-\Delta\Delta C_t = 3.56$), suggesting an association with endogenous degradation of red blood cells in clots.

3.2 HISTOPATHOLOGY

Despite the normal appearance of brain parenchyma, restricted hemorrhage and venous thrombosis were the hallmark findings in decalcified, paraffin embedded heads of rats exposed to a single shock wave with 2.9-17.5 peak overpressures. The sections stained with hematoxylin and eosin revealed consistent signs of hemorrhage and thrombosis in the subarachnoid space (including the choroid plexus) and the inner ear (Figures 5-13) after shock wave exposure. Table 1 shows the prevalence of these findings for low (2.9-7.9 psi [20-54.5 kPa] BOP) and medium (13-17.5 psi [89.6-120.7 kPa] BOP) intensity exposures; there are no significant differences in prevalence as a function of BOP magnitude (Fisher's exact tests, two sided).

TABLE 1. Prevalence of Hemorrhage and Venous Thrombosis

	2.9-7.9 psi BOP	13-17.5 psi BOP
Vertebrobasilar circulation	12/15 rats	16/17 rats
4th ventricle choroid plexus	8/15 rats	11/17 rats
Scala tympani and/or cochlear aqueduct	3/15 rats	9/17 rats
Hippocampal choroidal fissure	13/15 rats	10/17 rats
Velum interpositum including vein of Galen	11/13 rats	12/14 rats

3.2.1. Infratentorial findings

The vertebrobasilar circulation in the posterior fossa (Figures 5 and 8) and the choroid plexus of the fourth ventricle (Figure 6) showed signs of local hemorrhage and venous remodeling. The agglutinated erythrocytes and leucocytes were predominantly in the subarachnoid space, but could also be present in the subdural space at shorter (e.g., 24 hour) survival times and higher blast levels (Figure 5, 6 and 8).

The posterior fossa hemorrhages were associated with veins accompanying the basilar artery along the medulla and caudal pons, particularly near the junction of the vertebral and basilar arteries. These findings were remarkably persistent at later survival times; signs of hemorrhage were detected in the cisternal subarachnoid space even 42 days after blast exposure (Figure 5).

The choroid plexus of the fourth ventricle was another site of hemorrhage that did not vary in prevalence but varied in severity with BOP intensity. Only slight damage was observed in the 4.7-5.1 psi group, with small clumps of erythrocytes both on the ventricular surface of the plexus and in the cisterna magna (Figure 6 A-B). At higher intensities, the hemorrhage was more extensive. Figure 6C shows extensive hemorrhage in the choroid plexus near the proximal end of the foramen of Luschka, which contrasts sharply in appearance with the normal choroid plexus on the contralateral side.

Venous thrombosis was also found along the superior (anterior) aspect of the cerebellar cortex. Figure 7 shows one example of a thrombus (24 hours after BOP) that showed prominent interleukin 8 receptor B (IL8RB) immunoreactivity. Veins draining the tela choroidea of the fourth ventricle also could show a proliferation of vascular endothelial growth factor A (VEGFA)-immunopositive cells, accompanied by Col18a1 subunit expression in the choroid plexus. These findings were consistent temporally with the upregulation of respective mRNA (Figures 2 and 3); the differential location is consistent with local activation of angiogenic and angiostatic mechanisms during vascular repair.

The vertebrobasilar-derived vasculature of the inner ear also showed signs of hemorrhage in the perilymph compartment, which is confluent with the subarachnoid space through the cochlear aqueduct (Figure 8 CA). The prevalence was significantly lower than for vertebrobasilar damage in each exposure group (Fisher exact tests, $p < 0.02$), but did not differ from the prevalence of fourth ventricle choroid plexus damage. The hemorrhagic sites were almost exclusively in the scala tympani at the cochlear base, in association with the round window membrane near the opening of the cochlear aqueduct (Figures 8-10). This site is near the locus of the junction between the vestibulocochlear vein and the inferior petrosal sinus. The hemorrhages appeared to be more extensive after 13-17.5 psi BOP (Figures 8-9) than 4.7-5.1 psi BOP (Figure 10).

3.2.2. Supratentorial findings

Veins in the subarachnoid space showed consistent evidence of damage at some supratentorial sites. The veins in the choroidal fissure of the hippocampal formation showed signs of repair (Figure 11, middle panel) and thrombosis (Figure 11, lower panel). Persistent signs of hemorrhage were also observed in the neighboring subarachnoid space (Figure 11, upper panel). The coagulated regions in these veins contained IL8RB positive cells and the vessel walls showed collagen 8 alpha 1 subunit (endostatin precursor) immunoreactivity (Figure 12) at times when there was a corresponding mRNA

upregulation (Figures 2-3). These findings are consistent with blast damage followed by progressive vascular wound healing.

Immunohistochemical methods detected several products encoded by upregulated mRNAs in association with vasculature in the brain parenchyma of the BOP-exposed rats. Figure 11 (left upper panel) shows examples of procollagen 18 a1 subunit immunoreactive veins in the subarachnoid space and superficial frontal cerebral cortex, 24 hours after a 13 psi BOP exposure. Angiopoietin 1 immunoreactivity was associated with small blood vessels in both infratentorial and supratentorial regions. Examples of small caliber, Angiopoietin 1 immunoreactive vessels appeared in fiber tracts on the outer surface of the brain, such as the accessory optic tract at 24 hours (Figure 11, after 13 psi BOP) or 72 hours after the traumatic event (Figure 11, after 15 psi BOP). There was also a marked, time-dependent up-regulation of chemokine C-X-C motif receptor 3 in the internal capsule, extending into the white matter of the forebrain. The time course and intensity matches the profound changes in mRNA expression shown in Figure 4.

The veins of the third ventricle choroid plexus, smaller veins within the velum interpositum and the great vein of Galen showed evidence of thrombosis, even at low blast levels. An example of thrombosis in veins within the tela choroidea is shown in Figure 14A. The areas of thrombosis could extend rostrally toward the transverse fissure. More striking, though, was the thrombosis of small veins draining from the dorsal surface of the mesencephalon into the vein of Galen (Fig. 14 B), as well as clusters of agglutinated erythrocytes within the lumen of the vein of Galen (Fig. 14 C). These areas of thrombosis often extended from the sites of entry of small veins above the midbrain to the straight sinus. .

4. DISCUSSION

Experimental approaches to blast-induced mild traumatic brain injury can examine sequelae of primary blast injury, defined as direct effects of shock wave propagation through tissue. The blast

front forms the leading edge of the shock wave as an overpressure that propagates supersonically. The blast front is followed by a period of negative pressure termed the under-pressure [17]. These overpressure-underpressure blast wave sequences, termed Friedlander waves, propagate through the intracranial compartment as a positive-negative shift in intracranial pressure [18-20]. Transmission of a blast wave through the thoracic contents and great vessels to brain vasculature can also contribute in producing traumatic brain injury [21]. Microscopic hemorrhage, edema, engorgement of small veins and intravascular coagulation have been noted repeatedly in other tissues after blast wave exposure [8, 22, 23]. Analogous intracranial phenomena were demonstrated in decalcified heads in the present study.

The paraffin-embedded sections of decalcified heads provide valuable in situ preservation of vasculature and meninges that are disrupted by 'standard neuro-histologic procedure' of dissecting the brain from the skull before further tissue processing. These sections preserve even the most friable small veins and small areas of hematoma in the subarachnoid space. They also permit histopathological assessment of the velum interpositum, the junctions of intracranial veins with venous sinuses, and extensions of the cisternal space such as the cochlear aqueduct, Meckel's cave and the transverse fissure. Because the histological processing is compatible with immunohistochemical staining in a manner similar to any paraffin embedded section, the method can be used to elucidate molecular responses in the context of tissue biology. Thus, decalcified sections of the head offer considerable advantages in visualizing regions of vascular trauma that are otherwise discarded by standard practices. The appropriate range of exposures for animal models exploration of blast related mTBI is worthy of consideration. Perforation of the tympanic membrane (TM) has been proposed as a useful proxy indicator of peak shock wave exposures greater than 15 psi [24-26]. TM perforation is a common combat-related blast injury [24, 27-29], and there is a greater risk of loss of consciousness from combat blast exposures that perforate the TM [30]. Further, an acute study in combat reported that TM

perforation is a parsimonious model predictor of the number of mTBI symptoms [31]. Because experience from the field suggests that many individuals that are diagnosed with mTBI after blast do not suffer perforated ear drums [31], such injuries often occur below 15 psi exposures. Accordingly, this study used shock tube delivery of single, low level Friedlander waves (2.9 psi [20 kPa] - 17.5 psi [120.7 kPa] peak overpressure) to examine acute and subacute intracranial injury in rats. This range is explicitly lower than the overpressures (>17.5 psi/120 kPa) in many previous studies that have reported vascular and parenchymal injury in brains extracted from the skulls prior to histopathological assessment [32-44]. Biochemical changes have also been reported in similar ranges by others [45] [46].

The results of this study suggest that lower levels of blast wave exposure produce primarily vascular effects in rats. Messenger RNA profiles of the whole brains showed evidence of both blast intensity and time dependent effects on vascular wound healing markers. The rats exposed to 10-11 PSI overpressure tended to show a similar pattern of mRNA expression changes in these vascular repair and inflammatory pathways as rats exposed to approximately 5 psi overpressure, but the changes were greater at higher intensities. The changes in mRNA expression after a 14-15 psi exposure were different and suggestive of more severe injury, particularly for DNA repair, lymphocyte activation and lymphocyte migration pathways.

Histopathological examination of decalcified heads revealed that even 2.5-7.9 psi blast exposures produced a high prevalence of mild venous hemorrhage and thrombosis in the vertebrobasilar circulation, hippocampal choroidal fissure and the veins associated with velum interpositum. This phenomenon is consistent with reports of isolated and disseminated intravascular coagulation in both blast lung [47] and other forms of acute trauma, and possibly acts as triggers for mechanisms that can contribute to acute traumatic coagulopathy [48-50]. The isolated regions of intravascular coagulation in small veins and the isolated, very small venous hemorrhages in the subarachnoid space are worthy of consideration as factors in both healing and chronic sequelae of mild blast concussion. Although the

areas of local hemorrhage were small, remnants persisted in the subarachnoid space even 42 days after a single blast exposure. The high prevalence of very mild subdural and subarachnoid hemorrhage, below imaging detection thresholds, is a potential factor in the prevalence of post traumatic headache. The sequelae of larger subarachnoid hemorrhages include endothelin receptor-mediated vasospasm, microcirculatory spasm, microembolism and perihemorrhage ischemia [51], and may relate to 'early brain injury' by recruitment of apoptotic mechanisms [52, 53]. Hence, it is possible that local effects of small, shock wave-induced hemorrhages may play a role in producing more local vasospasm or activating apoptotic pathways that influence other chronic effects of mTBI and may play a role in some of the long term consequences. As such, the prevalence of subacute local intravenous coagulation raises the therapeutic consideration of managing, during the acute period, a possibly delicate balance between the degree of hemostasis required for wound healing and the local activation of fibrinolytic and anticoagulant mechanisms that have been identified in association with acute traumatic coagulopathy.

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FIGURES FOR RAT BOP paper

Figure 1. The left panel shows the orientation of the compression chamber and animal holding area of the shock wave tube at Naval Medical Center, San Diego. The apertures were placed between these portions of the tube and held a PVP membrane. The right panel shows examples of blast overpressure profiles that were measured inside (black) and extended tube and at the distal aperture of the tube (black) in another trial. Note the differences in the profile of the under-pressure component the follows the initial overpressure.

Figure 2. Changes in expression of whole brain mRNAs for products associated with fibroblasts. The results are displayed for survival times of 2, 24 and 72 hours after BOP exposure in three different peak overpressure ranges. The data are expressed as $-\Delta\Delta C_t$ relative to the control group. The line at $-\Delta\Delta C_t = 1$ represents a two-fold increase in expression of the particular mRNA.

Figure 3. Changes in expression of whole brain mRNAs for products associated with matrix metalloproteins and other basement membrane components, with fibronectins and integrins, and with promoters and inhibitors of angiogenesis. The results are displayed for survival times of 2, 24 and 72 hours after BOP exposure in three different peak overpressure ranges. The data are expressed as $-\Delta\Delta C_t$ relative to the control group. The line at $-\Delta\Delta C_t = 1$ represents a two-fold increase in expression of the particular mRNA; the line at $-\Delta\Delta C_t = -1$ indicates a two-fold reduction in expression.

Figure 4. Changes in expression of whole brain mRNAs for products associated with lymphocyte migration and with different chemokine pathways. The results are displayed for survival times of 2, 24 and 72 hours after BOP exposure in three different peak overpressure ranges. The data are expressed as $-\Delta\Delta C_t$ relative to the control group. Note that the profound changes the CXCR3 substrates are displayed on a logarithmic $-\Delta\Delta C_t$ scale, compared to a linear scale for other mRNAs. The line at $-\Delta\Delta C_t = 1$ represents a two-fold increase in expression of the particular mRNA; the line at $-\Delta\Delta C_t = -1$ indicates a two-fold reduction in expression.

Figure 5 Changes in expression of whole brain mRNAs for products associated with free radical metabolism, with cell cycle modulation, with proapoptotic TNF pathways, and with DNA repair proteins. The results are displayed for survival times of 2, 24 and 72 hours after BOP exposure in three different peak overpressure ranges. The data are expressed as $-\Delta\Delta C_t$ relative to the control group. The line at $-\Delta\Delta C_t = 1$ represents a two-fold increase in expression of the particular mRNA; the line at $-\Delta\Delta C_t = -1$ indicates a two-fold reduction in expression.

Figure 6. Photomicrographs from hematoxylin and eosin stained horizontal sections of decalcified heads. The upper left panel shows a region of subdural hemorrhage 24 hours after a single 13 PSI BOP exposure. The lower right and left panels show persistent evidence of residual hemorrhage in the subarachnoid space at longer survival time. An area of old hemorrhage persisted near the basilar artery at 42 days after a single 14.4 PSI BOP exposure (lower left panel). A hematoma in the subarachnoid space near the anterior pituitary (ant pit) is shown at lower and higher magnification in the right upper and lower panels, respectively.

Figure 7. Choroid plexus of the fourth ventricle 2 hours after either 5.1 psi (A-B) or 17.5 psi (C-D), BOP exposure. Note the areas of hemorrhage (arrows) in panels A-C.

Figure 8. Photomicrographs of both inner ears (left panels) and lateral medulla (right panels) from hematoxylin and eosin stained horizontal sections, 2 hr after a single 5.3 PSI blast wave exposure (rostral toward the right). Near the orifice of the cochlear aqueduct, agglutinated erythrocytes (arrows in left panels) were observed in the perilymph and in small veins near the RWM, but not in the middle ear cavity (ME). A small subarachnoid hematoma was present in the same sections (upper arrow, upper right panel), as well as thrombosis in superficial veins along the ventral half of the medulla and caudal pons (arrows in upper and lower right panels). Thrombosis was not observed more dorsally, rostrally or caudally.

Figure 9. Upper panels show a venous thrombus in the cisternal space anterolateral to the cerebellar hemisphere, 24 hours after single 13 PSI shock wave. The lower panels show a vascular endothelial growth factor A-immunopositive cell proliferation in a vein draining the tela choroidea of the fourth ventricle and Col18a1 subunit activity in the adjacent choroid plexus (24 hours after single 13 PSI shock wave).

Figure 10. . Lower power photomicrographs of the inner ear showing evidence of intrascalar hemorrhage at high blast intensities and longer survival times. Persistent evidence of hemorrhage was observed both in the intrascalar compartment near the cochlear aqueduct (CA) and within the CA.

Figure 11. The left column shows H&E sections of a thrombus containing erythrocytes, exudate and infiltrating nucleated cells (E&I) in the subarachnoid space (upper panel). Normal choroidal veins and arteries are shown after a 13 PSI peak exposure in the middle left panel, but blood clots associated with exudate and infiltrating nucleated cells were observed near the choroidal vessels after a 15 PSI peak shock wave (lower left). The right column shows representative immunohistochemical results. Procollagen 18 a1 subunit immunoreactive veins in the subarachnoid space and superficial frontal cerebral cortex are shown in the upper right panel after a 13 PSI peak shock wave exposure. The right middle panel shows angiopoietin 1 immunoreactive small blood vessels in the accessory optic tract after 13 PSI peak exposure. The right lower panel shows more intensely angiopoietin 1 immunoreactive vessels in a similar location 72 hours after 15 PSI peak exposure.

Figure 12. Blood vessels in the choroidal fissure, 24 hours after single 13 PSI shock wave. A section stained with hematoxylin and eosin (H&E) is shown in the upper left panel. Note the clot formation (microthrombi resembling a platelet-plasma clot), with IL-8 positive cells (upper right panel), associated with a large vein and the association of Col18A1 expression with vessels (lower panels).

Figure 13. Immunohistochemical results showed upregulation of receptor expression angiostatic chemokine C-X-C motif receptor 3 mechanisms after single BOP exposure. The expression was associated with blood vessels in the internal capsule (shown here). The time course and intensity matches the changes in mRNA expression shown in Figure 4.

Figure 14. Photomicrographs of intravenous thrombosis 2 hours after a single 4.9 PSI BOP exposure from horizontal sections of a decalcified head. The micrographs are taken near the midline and oriented such that the rostral direction is rightward. The arrows in panel A indicate regions containing coagulated erythrocytes within the internal cerebral vein and, more laterally and caudally, the basal vein of Rosenthal. Note the normal choroid plexus of the third ventricle along the midline. Panel B shows cross-sectional views of small, thrombotic veins between the inferior colliculi and the superior colliculi (Sup Coll). Panel C shows a coagulated mass of erythrocytes within the great cerebral vein of Galen.