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Prolonged but not short duration blast waves elicit acute inflammation in a rodent model of primary blast limb trauma

Keywords: Rodent model; blast limb trauma; inflammatory response

Abstract

Background

Blast injuries from conventional and improvised explosive devices account for 75% of injuries from current conflicts; of these over 70% involve the limbs. Variable duration and magnitude of blast wave loading occurs in real-life explosions and is hypothesised to cause different injuries. While a number of *in-vivo* models report the inflammatory response to blast injuries, the extent of this response has not been investigated with respect to the duration of the primary blast wave. The relevance is that explosions in open air are of short duration compared to those in confined spaces.

Methods

Hind limbs of adult Sprauge-Dawley rats were subjected to focal isolated primary blast waves of varying overpressure (1.8-3.65kPa) and duration (3.0-11.5ms), utilising a shock tube and purpose built experimental rig. Rats were monitored during and after blast. At 6 and 24hrs after exposure blood, lungs, liver and muscle tissue were collected and prepared for histology and flow cytometry.

Results

At 6hrs increases in circulating neutrophils and CD43Lo/His48Hi monocytes were observed in rats subjected to longer duration blast waves. This was accompanied by increases in circulating pro-inflammatory chemo/cytokines KC and IL-6. No changes were observed with shorter duration blast waves irrespective of overpressure. In all cases, no histological damage was observed in muscle, lung or liver. By 24hrs post-blast all inflammatory parameters had normalised.

Conclusions

We report the development of a rodent model of primary blast limb trauma that is the first to highlight an important role played by blast wave duration and magnitude in initiating acute inflammatory response following limb injury in the absence of limb fracture or penetrating

31 trauma. The combined biological and mechanical method developed can be used to further
32 understand the complex effects of blast waves in a range of different tissues and organs *in-vivo*.
33

34 **Introduction**

35 Blast injuries from conventional and improvised explosive devices (IEDs) account for 75% of
36 modern war injuries, over 70% of these involve the limbs [1]. Blast injuries remain a threat to
37 civilians too; detonation of IEDs in the recent Boston marathon bombings in 2013, together
38 with industrial accidents such as the 2013 west Texas fertilizer plant explosion caused many
39 injuries with a similar pattern to that seen amongst military casualties [2].

40 Blast trauma may occur by four discrete mechanisms: primary injuries are due to the interaction
41 between the blast wave and the human body, secondary injuries are caused by the impact of
42 fragments thrown and energised during the explosion, tertiary injuries result from acceleration
43 of the body against an obstruction, and quaternary injuries include other physical insults, such
44 as burns and smoke inhalation [3-5]. The type and severity of the injury sustained depends on;
45 the explosive system and the environment of the blast; the size of explosive device, the distance
46 between the person and the explosion and the presence of obstacles or reflections. In an open
47 space (free field blast) a blast wave spreads radially from its origin and quickly dissipates as a
48 function of the cube of the distance [3]. In these cases the blast wave consists of a rapid rise to
49 a positive overpressure followed by a negative under-pressure and return to ambient pressure.
50 In an enclosed space, the explosive energy is contained leading to rises both in the peak
51 overpressure and the duration of the positive-pressure phase of the blast wave [3]. Previous
52 studies have shown that explosions within enclosed spaces are associated with a higher
53 incidence of primary blast injuries and more severe injuries compared to open air explosions
54 [6].

55 In both the civilian and military setting, blast injured patients are often poly-traumatised with
56 the head, torso and soft tissues commonly affected [7-8]. The understanding within the trauma
57 community of the relationship between injury, inflammation, sepsis and clinical outcome is
58 growing [9-11]. It is important to note that blast injury survivors may suffer shock or
59 hypoxemia in the absence of external signs of injury [12]. The onset of inflammation following
60 injury is a common phenomenon, however, in severely compromised patients (particularly
61 those with sepsis) systemic inflammation may contribute towards deleterious and life
62 threatening changes, such as multi-organ failure, which are difficult to manage clinically [11].

63 *In - vivo* animal models are often used with simulated blast conditions in a controlled
64 environment to investigate the mechanisms of blast injury. These studies enable greater

65 understanding of the nature of the injury, including mechanical failure thresholds of tissues,
66 physiological or inflammatory responses, and the effect of therapeutic interventions. However,
67 given the clinical burden of lower limb blast injuries [13], few experimental live models have
68 been reported relating to blast limb trauma; those undertaken show that explosive limb injury
69 may lead to systemic inflammatory changes affecting the limbs as well as distal organs [14-
70 17]. However, the injury documented in these models is severe and encompasses several blast
71 injury mechanisms. It is recognised that further research is needed to closely examine the
72 interplay of different blast mechanisms in limb injury [18].

73 During IED explosion, blasts with peak pressures from 50 to 1000 kPa and 2-6 ms duration
74 have been measured [19]. However, the majority of the experimental animal models involve
75 blast waves with longer durations between 4 – 8 ms [20-22] and some with durations longer
76 than 10 ms [23]. A large number of existing models also lack detail when reporting the pressure
77 histories of the blasts produced, with many reporting only the peak overpressure or the distance
78 from the outlet of the shock tube, often without details or schematics of animal positioning and
79 orientation, thus limiting comparability between studies [24-25].

80 In this study we develop a model to investigate the inflammatory response to primary blast
81 wave application to the limb, investigating the effect of changing the magnitude or duration of
82 the blast wave, thus permitting the controlled delivery of primary blast to replicate durations
83 associated with a range of open field and enclosed environments.

84

85 **Materials and Methods**

86 *Characterisation of the Injury Device*

87 A shock tube (Figure 1A) was employed in this study to generate pressure pulses of controlled
88 intensity and duration. The shock tube used is a stainless steel tube 3.8 m long and 59 mm in
89 internal diameter. It is comprised of two chambers; the first chamber, the driver section, is filled
90 with compressed air [26] and the breech releases the pressure into the driven, low-pressure,
91 section producing a shock wave [27].

92 In order to characterise the blast waves generated, three dynamic pressure gauges were installed
93 along the shock tube's driven section. The first two pressure gauges were orientated radially at
94 the middle (Sensor 1) and end of the driven section (Sensor 2) as shown in Figure 1A. The
95 third (Sensor 3) was installed on a bull-nose probe of 2 cm in diameter sited axially facing
96 upstream. Sensor 1 was used for triggering and monitoring the reproducibility of the blast.
97 Sensor 2 for monitoring the output pressure, and Sensor 3 was used to characterise the pressure
98 pulse at the sample position.

99 While the magnitude of the shock wave generated can be altered by using diaphragms of
100 different burst pressure, the duration of the wave is controlled by changing the length in the
101 driver tube. The 10% driver volume uses only the volume of the breech system (Figure 1A)
102 while, 100% volume uses the full length of the driver tube. It should be noted that the 10%-
103 volume driver generates the Friedlander waveform of open-air blasts, while full-volume driver
104 produces blasts of longer pulse width (duration), similar to those seen in large-charge
105 explosions and enclosed volumes such as inside a vehicle.

106 With the *in-vivo* model the sample replaced Sensor 3. Sensor 2 was then used to monitor the
107 loading pressure. Three different experimental conditions were used:

108 200-µm Mylar® diaphragm, bursting at ~ 15.7 ± 0.5 bar with 10% driver volume

109 75-µm Mylar® diaphragm, bursting at ~ 6.2 ± 0.2 bar with 10% driver volume

110 50-µm Mylar® diaphragm, bursting at ~ 6.0 ± 0.1 bar with full (100%) driver volume

111 *Induction of injury*

112 Sprague-Dawley (*Rattus norvegicus*) female rats weighing 250-320 grams were used. All
113 animals were housed in clean cages and kept on a 12-hour light/- dark cycle with unlimited
114 access to food and fresh water. All animals were anaesthetised with 60 mg/kg ketamine and
115 0.20 mg/kg medetomidine [28] administered intraperitoneally and randomly allocated into four
116 groups as summarised in Table 1. Sham animals were anaesthetised but not exposed to a blast
117 wave. Animals from Groups I and II were anaesthetised and exposed to a single blast insult of
118 6.0 and 16.0 bar overpressure respectively. Animals in the final Group III were exposed to a
119 6.0 bar blast wave of a longer duration (Table 1) compared to animals in Groups I and II.

120 **Table 1: Experimental Groups used in the study**

121 A purpose-built subject support (Figure 1B) was developed to expose the rat's hind limbs to a
122 focussed primary blast wave, whilst ensuring the remainder of the animal was not exposed.
123 This was done to ensure local loading and to prevent the abdomen and chest sustaining injury.
124 The animal's hind limbs were then strapped using adhesive tape to a steel bar in order to prevent
125 limb movement and avoid tertiary blast effects.

126 **Figure 1: (A) Shock tube schematic. (B) Experimental rig mounted at the outlet of the shock**
127 **tube to isolate the blast to the animal's left hind-limb. The distance from the shock tube's**
128 **outlet to the left thigh is 5cm. The left leg is exposed to the shock wave from the pelvis to**
129 **the ankle joint.**

130 All animals were closely monitored after recovery and received buprenorphine (0.03 mg/kg
131 administered subcutaneously) for analgesia as a precaution. For the remainder of the study, the
132 animals were monitored 3 times a day in their facility for several behavioural parameters,
133 including appearance, weight changes, and response to handling. Six animals from the Sham
134 group and Groups I, II and III were observed for 6 hrs to investigate the acute inflammatory
135 response; in line with the 3R's principle; Shams, Group I and Group III were observed up to
136 24 hrs to track the resolution of inflammation.

137 *Data collection and analysis*

138 Physiological parameters

139 Heart rate data were collected using a Veterinary Handheld Oximeter (Creative Medical,
140 Shenzhen, China) at 1 min and 5 min after induction of anaesthesia, and then 1 min and 5 min

141 after blast or sham procedure. Finally, heart rate data were also collected once the subjects
142 recovered (40 min after the initial induction of anaesthesia).

143 Collection of blood and plasma

144 At specified time points of 6 or 24 hrs, rats were intraperitoneally (i.p) injected with an
145 overdose of Pentobarbitone and blood was collected in citrated tubes on ice from the right
146 femoral vein and centrifuged at 2500 rpm for 10 mins. Plasma was collected and stored at -80°C
147 for further analysis.

148 Blood processing and flow cytometry

149 Red blood cells were lysed in ammonium chloride buffer and centrifuged for 10 min at 2500
150 RPM. Cell pellets were re-suspended and viable cells were counted using Trypan blue staining
151 solution on a haemocytometer (Nikon, Tokyo Japan). Flow cytometry was performed using a
152 method adapted from Barnett-Vanes et al., (2015). Briefly, cells were stained with live-dead
153 stain (eBioscience), blocked with anti-cd32 blocking antibody and stained with antibodies
154 CD43 PE (Biolegend) and His48 FITC (Biolegend) for neutrophils and monocytes, CD161
155 APC (Biolegend) for NK Cells and CD3 VioGreen (Miltenyi Biotec) for T Cells, in buffer
156 containing PBS, BSA and Azide [29]. Cells were fixed in BD Cell Fix and analysed using a
157 multi-parameter flow cytometer (Fortessa LSR BD Biosciences New Jersey USA). Flow
158 cytometric compensation was performed using fluorescent compensation beads (OneComp
159 eBeads, eBioscience San Diego USA).

160 Cytokine analysis

161 Blood plasma was thawed and analysed using a multiplex ELISA (MesoScaleDiscovery
162 Maryland, USA) according to the manufacturer's instructions. A microtitre plate pre-coated
163 with capture antibody was incubated with blocking buffer at room temperature (RT) with
164 agitation for 1 hour. After washing, standards or samples were added and the plate was
165 incubated at RT with shaking for 2 hours. The plate was then washed and incubated with
166 detection antibody conjugated to electrochemiluminescent labels at RT for 2 hours. After
167 washing, read buffer was added and the plate was read on an MSD plate reader.

168 Histology

169 Liver (Right Lateral Lobe), lung (left lobe) and left quadriceps muscle samples were harvested
170 and fixed for 24hrs in 10% Buffered Formalin. Paraffin-embedded sections (4 μm) were stained
171 with hematoxylin and eosin (H&E). Images of the slides were captured using a Leica light
172 microscope (Wetzlar, Germany).

173 Statistics

174 All data were collected from 2-4 individual experiments. Mechanical data are expressed as
175 mean ± standard deviations (SD). Physiological parameters were compared using a two way
176 repeated measures Analysis of Variance (ANOVA) test using SPSS (version 22.0, Chicago,
177 USA). All other data were analysed using a non-parametric Mann Whitney t-test expressed as
178 mean ± Standard Error of Mean (SEM) using GraphPad Prism v5 (San Diego, USA), *$p<0.05$,
179 **$p<0.01$.

180

181 **Results**

182 The characteristics of the blast waves produced in the shock tube are described by mean burst
183 and corresponding shock wave peak pressures, plateau pressure, and impulse duration from
184 Sensor 3 for all three test configurations (Table 2).

185 **Table 2: Average burst pressure, corresponding peak pressure, plateau pressure, and shock**
186 **impulse (relative to ambient pressure) from Sensor 3 for three loading scenarios (mean and**
187 **standard deviations for each condition are from 3 individual experiments).**

188 Sensor 2 traces show that all three waves exhibit a typical pattern consisting of a positive
189 overpressure followed by a negative under-pressure and subsequent return to ambient pressure
190 (Figure 2A). Sensor 3 gave slightly higher amplitudes, faster rise times and longer durations
191 than the tracings of Sensor 2. The internal stress produced in the sample by a blast is influenced
192 by the mechanical impedance of the sample, so measurements from the sensor inside the bull-
193 nose that has a diameter one third the internal diameter of the shock tube [27-26] set the pressure
194 trace's upper limit and enable readings more relevant to small samples than Sensor 2 regarding
195 the conditions under which the specimens are loaded. In the absence of Sensor 3 the second
196 lateral sensor was used to identify the loading pressure magnitude. A linear relationship
197 between the peak pressures measured by Sensors 2 and 3 (Figure 2B) enabled estimation of the
198 shock magnitude reaching the subject when only Sensor 2 was used.

199 **Figure 2: (A) Traces from Sensors 2 and 3 (relative to ambient pressure) of three loading**
200 **configurations and (B) Relationship between the peak pressures (relative to ambient**
201 **pressure) measured by Sensor 2 and Sensor 3.**

202 The repeated measures ANOVA test determined that there were no statistical differences in the
203 heart rates measured between the four Groups ($p=0.842$) across the different time points that
204 the measurements were obtained.

205 At 6hrs significant increases in circulating blood neutrophils were seen in Group III (mean
206 2.6E+06, SEM 0.360E+6) compared to Shams (mean 0.744E+6, SEM 0.161E+6), Groups I
207 (mean 0.674E+6, SEM 0.164E+6) and II (mean 0.540E+3, SEM 0.085E+6). Circulating
208 monocytes were significantly increased in Group III (mean 162E+3, SEM 21.8E+3), in
209 comparison to Shams (mean 40E+3, SEM 10.1E+3) and Group II (mean 38.8E+3, SEM
210 4.60E+3). By 24hrs, no significant differences were observed between sham and blast groups
211 (Figure 3A). No significant differences were observed in the proportion of CD43Hi/His48Int-
212 Lo, NK Cell or CD3 T Cells between groups at either timepoint (Figure 3B).

At 6hrs significant increases in circulating IL-6 were seen in Group III (mean 223, SEM 74.5) compared to Shams (mean 34.4, SEM 8.94) and Group I (mean 40.5, SEM 7.19). Increases were also seen in circulating CXCL1 in Group III compared to Shams but this was not significant (p=0.125). By 24hrs, no differences were observed in IL-6 or CXCL1 between sham and blast groups (Figure 3C).

Figure 3: (A) Shows the number of circulating neutrophils and CD43Lo/His48Hi monocytes observed at 6hrs and 24hrs for all groups. Representative flow cytometry plots are shown for Sham and Group III at 6hrs. (B) Proportions of circulating CD43Hi/His48Int-Lo monocytes, NK Cells and T Cells were examined. (C) Representative bar graphs showing the concentration of circulating IL-6 and CXCL1 in the plasma. Data are n=4-6, performed in duplicate from 2-3 separate experiments, *$p<0.05$, **$p<0.01$.

No macroscopic changes were seen by histology in limb muscle, liver or lung using H&E staining at 6hrs in Groups I or III (Figure 4).

Figure 4: Histological sections of muscle, lung and liver tissue. Quadriceps muscle, left lung lobes and superior right lateral liver lobes were fixed in formalin, embedded in a wax block, cut and stained with Hematoxylin and eosin (H&E) stain. Slides were imaged under a light microscope at 10X magnification.

Discussion

The development of a rodent model of primary blast limb trauma using a compression driven shock tube to deliver an isolated and controlled blast wave is reported. To our knowledge, it is the first study to examine the relationship between the effects of long versus short duration primary blast waves on the systemic inflammatory response. This has relevance in the field to open (free field) versus enclosed (in-vehicle or building) blast waves respectively.

Blast Waves and Injury

Blast waves generated by explosive devices may cause severe damage by coupling energy into human tissues, initiating onward stress wave propagation [30]. Due to the destructive capability of these waves, particularly at interfaces of differing tissue densities, organs such as the lungs and bowel are thought to be most susceptible to damage. Studies show the injuries sustained during explosions are significantly influenced by their environment. In open air blasts, fragments that are part of the explosive device (primary fragments) or from the explosion (secondary fragments) are responsible for the majority of injuries sustained [6]. However,

244 victims of bombings that occur in confined spaces suffer a higher incidence of primary blast
245 injuries, which are more severe and result in clinically worse conditions [6]. For example, a
246 significantly higher rate of primary blast lung injury (PBLI) is seen amongst mounted/in-
247 vehicle casualties compared with those dismounted following an external explosion [31].

248 *Animal Models*

249 In experimental studies of primary blast, animals are usually securely fixed to an animal holder,
250 often using a metal mesh plane or plate [20].This may be suspended within the main section
251 [20-21, 32] or across the outlet of the shock tube at varying distances and body orientations,
252 with or without a reflective plate [33-34] thus, leaving most of the subject's body exposed to
253 the blast wave. In certain reports, particularly in studies of traumatic brain injury (TBI), a
254 Kevlar vest [35] or 'body armour' [34] is utilised, wrapped around the animal's thorax to
255 protect the animals from blast lung injury whilst leaving the head fully exposed. Because of
256 concerns surrounding thoracic loading despite the presence of Kevlar protection, and the
257 danger of blast waves pressuring an enclosed protective cylinder, our model utilises a purpose
258 built experimental rig and harness to deliver isolated shock waves to the left hind limb of a
259 rodent. However, whilst blast waves have a complex form which varies both in intensity and
260 duration, most blast research on biological systems has focussed overwhelmingly on shock
261 overpressure, neglecting the effect of pulse duration which remains poorly understood.

262 *Physiological Response*

263 Studies of blast injury to the thorax show a transient bradycardia following blast as a result of
264 a vagally driven depressor reflex [36-37]. Though previous studies have recorded shockwaves
265 developed after missile extremity impact propagating to the abdomen, thorax and brain in
266 anesthetised animals [38-40], our observation of no significant differences in heart rate after
267 blast limb injury - in addition to the lack of histological findings - supports our assertion that
268 the thorax was not exposed to blast loading. This, therefore, is a true 'isolated blast' model.

269 *Inflammation*

270 Flow cytometry was performed to dissect the inflammatory response and it was found that in
271 in group III there was a significant increase in circulating neutrophils and CD43 Lo/His48Hi
272 (Classical) monocytes compared to Shams (Figure 3A) a response that was not seen in groups
273 I and II. No significant changes were observed in the % of CD43Hi/His48Int-Lo (non-classical)

274 monocytes, NK cells or T Cells in the blood of rats following blast (Figure 3B). These results
275 indicate that the systemic inflammatory response is correlated with the duration of the blast
276 wave rather than the extent of the overpressure. Characterisation of the nature of the cellular
277 inflammatory response showed a selective increase in neutrophils and CD43 Lo/His48Hi
278 monocytes consistent with previous reports of rats responding sub-acutely to damage or
279 inflammatory signals [41-45].

280 Alongside cellular inflammation, at 6 hours we observed raised levels of the inflammatory
281 cytokines CXCL1 and IL-6 in Group III compared to Shams that was not seen in Groups I and
282 II suggesting again that the extent of inflammation is correlated with duration rather than the
283 magnitude of the blast overpressure. Of note CXCL1 has previously been shown to mediate
284 neutrophil mobilization from the bone marrow-directly in a model of hind limb perfusion [46]
285 and in a model of blast lung injury [47-48], suggesting that the enhanced levels of CXCL1 seen
286 in this model may account for the increase in circulating neutrophils. By 24hrs, both mediators
287 had returned to basal levels. Of note and in contrast with other studies of blast limb trauma,
288 [16] we observed no substantive macroscopic changes in limb muscle, liver or lung muscle at
289 6hrs (Figure 4), supporting our method that blast waves were only applied to the limb and did
290 not inadvertently damage other organs which would complicate the inflammatory response.
291 Moreover, we saw no limb fractures in this model, likely due to the absence of flail against a
292 hard surface.

293 Recent studies of more complex models [49-50] have outlined the importance of primary blast
294 effects in exasperating other injuries and delaying recovery. Moreover, increasing use of novel
295 explosives which generate blast waves of longer duration necessitates a better understanding
296 of their impact on injury.

297 In this study, we have developed an experimental set up that allows us to accurately study the
298 effects of short duration (free-field) and long duration (enclosed spaces) blast of specified
299 magnitudes in an *in vivo* model. We have shown that focal application of prolonged but not
300 short duration blast waves to a single hind limb promotes acute increases in circulating
301 neutrophils and CD43Lo/His48Hi monocytes, together with rises in pro-inflammatory
302 cytokines in the absence of any visible gross tissue damage. This research points to an
303 important role played by blast waves in initiating inflammatory changes in the absence of limb
304 fracture or penetrating injuries. It is well known that sub-lethal trauma can exacerbate the
305 inflammatory response to secondary inflammatory stimuli including infectious agents or

306 mechanical stimuli, such as lung ventilation [11 51]. This study shows that the immune system
307 is exquisitely sensitive to the greater damage induced by blast overpressures of prolonged
308 duration.

309 *Conclusions*

310 This study demonstrates focal application of blast waves to the limb can elicit a systemic
311 inflammatory response characterised by changes in circulating inflammatory cells and
312 cytokines. The changes observed are; dependent on the characteristics (notably duration) of the
313 wave, relatively short-lived (normalising by 24 hours), and occur in the absence of other blast
314 wave injury mechanisms. Furthermore, the combined biological and mechanical method
315 developed in this article is a significant step to further our understanding of the complex effect
316 of blast waves in a range of different tissues and organs *in-vivo*.

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327

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