EPJ Web of Conferences **26**, 03006 (2012)
DOI: 10.1051/epjconf/20122603006


# Pressure pulse induced-damage in live biological samples

C. Bo[1,a], J. Balzer[1], S. Godfrey[2,3], M. Francois[2], J.L. Saffell[3], S.M. Rankin[2], W.G. Proud[1], and K.A. Brown[1,4]

[1] *Institute of Shock Physics, Imperial College London, London SW7 2AZ, UK*
[2] *National Heart & Lung Institute, Imperial College London, London SW7 2AZ, UK*
[3] *Division of Brain Sciences, Imperial College London, London W12 0NN, UK*
[4] *Institute for Cellular and Molecular Biology, University of Texas, Austin TX 78712, USA*

**Abstract.** Developing a cellular and molecular understanding of the nature of traumatic and post-traumatic effects of blast on live biological samples is critical for improving clinical outcomes. To analyze the effects of blast waves upon the cellular structures and the underlying physiological and biochemical changes, we have constructed an experimental platform capable of delivering compression waves, of amplitudes relevant to blast, to cell suspensions in a contained environment. Initial characterization of the system shows that cell cultures can be subjected to high-intensity compression waves up to 15 MPa in pressure and duration of $80 \pm 10\,\mu$s. Studies of mouse mesenchymal stem cells subjected to two different pressure impulses were analysed by cell counting, cell viability assays and microscopic evaluation: the experiments present evidence suggestive of increased levels of damage and loss of cellular integrity compared to uncompressed cell cultures.

## 1 Introduction

The physical processes involved in the body's response to a blast event are very complex. Due to the sudden increase in pressure on the body, organs with high air content are compressed and tissue damage occurs when the strength of the tissue is overcome [1]. Amongst the factors that can influence the type of injuries produced there are peak overpressure in conjunction with its duration (impulse), the environment in which the explosion occurs (free field versus confined space), wave form, body mass and the proximity of the target to the explosions [2]. Blast injuries due to exploding missiles were first classified by Zuckerman [3] into four groups: Primary Blast Injuries, resulting from exposure of the body to pressure variations accompanying the blast wave; Secondary Blast Injuries, penetrating and non penetrating injuries due to fragments propelled by blast force; Tertiary Blast Injuries, caused by the acceleration of the entire body against mobile or fixed objects; Quaternary Blast Injuries, all other injuries not covered by the previous groups such as chemical poisoning, burns, etc. Data collected from conflict zones have shown that the majority of the casualties of bombings present injuries in the extremities [4]. Improvements in body armour design, enhanced pre-hospital care and rapid aeromedical evacuation have led to a significant decrease in the number of fatalities in modern conflicts [2]; indeed there was an increase in survivability from 69.7% during WWII to 88.6% in the most recent conflict in Iraq [5]. This increase in survivability has been accompanied by an increase in casualties with complex injuries, associated in particular with the use of Improvised Explosive Devices (IEDs). These blast-associated traumatic injuries are characterised by multiple fractures [6] and chronic pain, often leading to amputation. These injuries are invariably as a result of high strain rate deformation of both hard and soft tissues, resulting in fracture patterns, injury zones and tissue dysfunction that are uncommon in civilian injuries [7]. Complications associated with these types of traumatic blast-associated injuries include heterotopic ossification (HO, aberrant bone formation near the wound site) and increased incidence of bone infections (osteomyelitis) [8,9]. Improving treatments for these complications requires a better understanding of cellular and molecular changes that occur in traumatized tissues.

Here we present a primary blast experimental model, designed to subject mesenchymal stem cells (MSCs) suspensions to pressure pulses. A compressive split Hopkinson pressure bar (SHPB) setup has been modified adding a confinement chamber that hosts the biological samples during the compression experiments. The current design allows recovery of the cell suspensions after compression for subsequent cellular analysis. Initial results indicate that approximately 80% of the liquid sample can be recovered. To ensure biocompatibility, control samples, refered to as uncompressed, were subjected to the same handling processes as all the other samples, except they were not subjected to compression in the SHPB: no evidence of cellular defects were detectable in uncompressed samples. Changes in cellullar viability and integrity are observed in cultures subjected to compression.

## 2 Materials and methods

### 2.1 Experimental setup

The SHPB system used in the compression experiments (Cavendish Laboratory, University of Cambridge) was composed of four bars made from Inconel steel. The striker bar was 12.7 mm in diameter and 180 mm in length while the input, output and momentum trap bars were 12.7 mm in diameter and 500 mm in length. A confinement chamber, previously described in [10,11], was mounted on the input

[a] e-mail: c.bo10@imperial.ac.uk

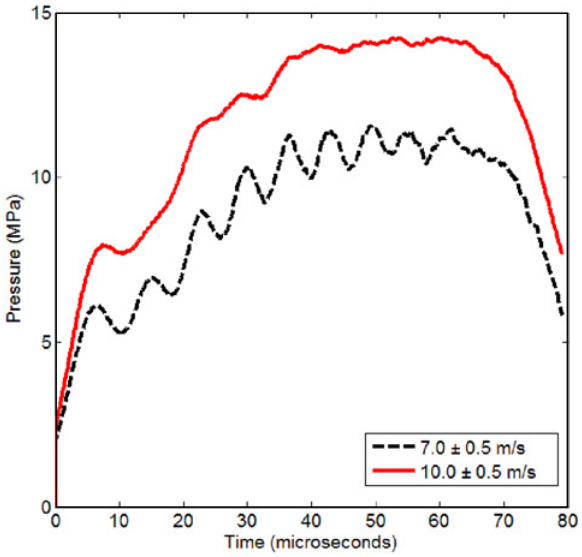


**Fig. 1.** Pressure pulses corresponding to two different impact velocities of the striker bar.

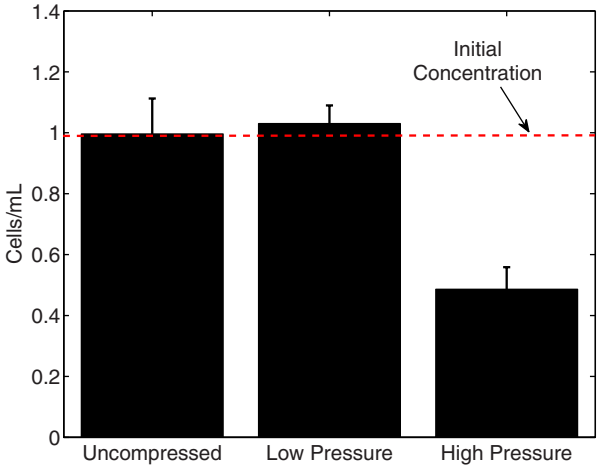


**Fig. 2.** Average values of cell concentrations measured on three groups of 5 samples, uncompressed, compressed at low pressure and compressed at high pressure, respectively. Average cell concentrations from each sample group prior to placement in the chamber are shown by the dotted red line.

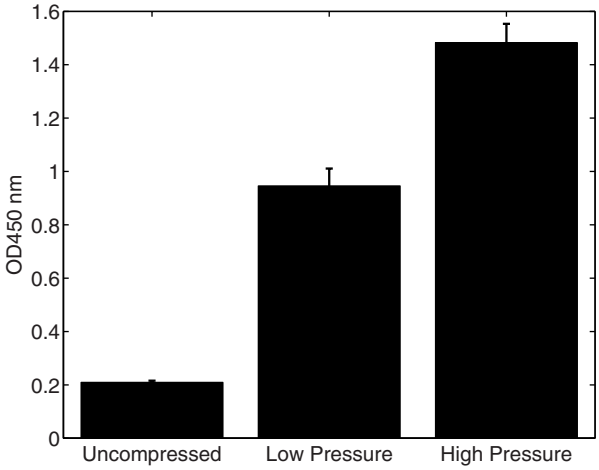


**Fig. 3.** Average values of $OD_{450nm}$ units, indicative of relative levels of LDH present in supernatants (5 samples each) from uncompressed MSCs, and MSCs compressed at low pressure or high pressure conditions.

and output bar to contain the biological samples during compression. Briefly, the chamber consisted of three parts, a main hollow cylindrical body in polycarbonate that slides on the two bars with two bore holes where the liquid was inserted using a syringe; two polycarbonate discs with O-ring seals; and two brass screw caps that hold the components together and align the chamber on the bars. Once the sample was inserted the bars were gently pushed togeter to eliminate air bubbles and a metal screw was inserted to seal the top of the chamber.

The strain of the bars was recorded using semi-conductor gauges (Kulite type AFP 500-90) in order to determine the pressure P as a function of time in the liquid, defined as:

$$P(t) = \frac{A_0}{A_s} E_0 \varepsilon_t(t) \tag{1}$$

where $A_0$ is the bar cross section area, $A_s$ is the surface area of the fluid column, $E_0$ is the Young's modulus of the bars and $\varepsilon_t$ is the transmitted bar strain as defined in classical SHPB theory [12].

### 2.2 Biological samples

Mesenchymal stem cells (MSCs) are a heterogeneous population of self-renewing and multipotent stem cells that can differentiate into a variety of mesenchymal lineages (i.e. bone, fat, cartilage) [13]. They usually reside in the bone marrow but they have been found also around blood vessels in various tissues as muscle tissue, adipose tissue and epidermal tissue [14]. Several studies have identified bone progenitor cells, including MSCs, as potentially contributing to the development of HO following blast injury [9]. MSCs have also been shown to migrate and home to sites of injury, and it has been speculated that injured cells could also release chemotactic signals [15]. MSCs could hence play a key role in the repair and/or disfunctional processes that follow blast injuries.

For the experiments described here mouse femurs and iliac bones were digested in a Collagenase type I and II buffer to extract MSCs. Cells were then seeded in tissue culture flasks and expanded to near-confluence (70%–90%) at 37 °C, 5.0% $CO_2$. MSCs were split after trypsinization (0.05% Trypsin/ethylenediamine-tetraacetic acid, Invitrogen) and replated in fresh media. The cells used in the compression experiments were at passage three (i.e. after three weeks in cell culture). The media used to expand the cells consisted of 80% Dulbecco's Modified Eagle Medium (Life Technologies), 10% Fetal Bovine Serum (Life Technologies), 1% penicillin-streptomycin (Invitrogen), basic fibroblast growth factor and heparin (1 U/mL). The chamber and the bars were cleaned with 70% ethanol and allowed to dry before each experiment. All the samples were resuspended by gently pipetting into a microfuge tube, to ensure a homogenous distribution of material prior to loading in the sample chamber. 1-mL

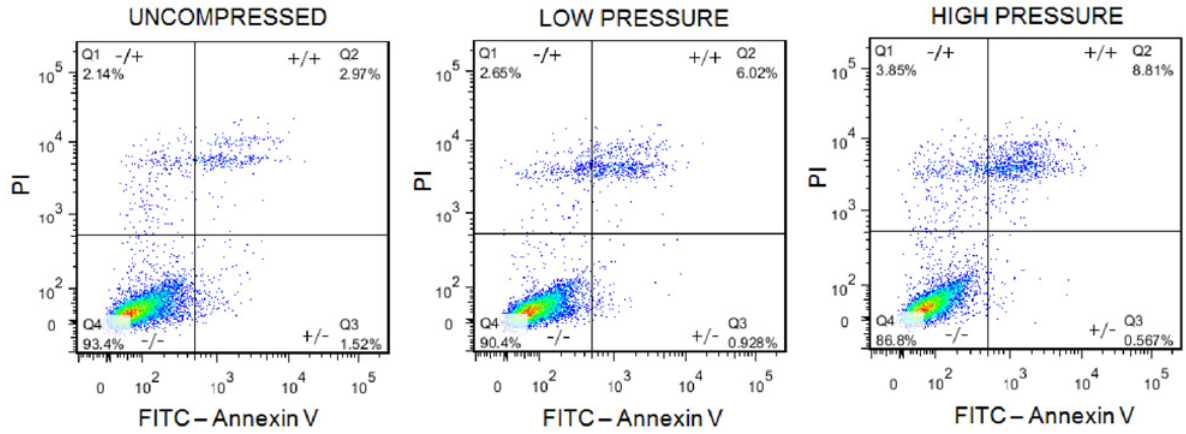


**Fig. 4.** FACS analysis of uncompressed and compressed samples stained with FITC-Annexin V/PI. Only two populations of cells are detectable in these graphs: PI negative cells, which are live cells with an intact plasma membrane and occupy in the graphs quadrants Q3 and Q4 and PI positive cells, which are dead cells intrepreted as having a damaged plasma membrane since they are unable to exclude PI, corresponding to events gated in quadrants Q1 and Q2.

sample aliquots, at concentration of approximately $10^6$ cells per mL, were generated by placing 1-mL of resuspended cells culture in 1.5-mL microfuge tubes prior to carrying out experiments using the SHPB. Five aliquots (uncompressed) were each inserted and then removed from the chamber, and then each placed in a new microfuge tube. Two additional groups of five 1-mL aliquots (compressed at low pressure and high pressure, respectively) were each inserted into the chamber, subjected to a compression wave using the SHPB system with impact velocities of 7.0 ± 0.5 m/s or 10 ± 0.5 m/s. Each sample was then removed and placed in a new microfuge tube. The chamber was rinsed with fresh culture medium before and after each aliquot of cells was inserted. The samples were analysed within 4 to 6 h after compression. First, cell concentration of the samples was measured by diluting 10 $\mu$L of each sample in methylene blue (dilution factor 3) and transferring then 10 $\mu$L of the dilution to a counting slide. Four squares were selected to count the number of cells in the slide, excluding the cells that touched the edges of the grid. The concentration was determined as: cells permL = average count per square x dilution factor $\times 10^4$.

Each microfuge tube was then centrifuged at 160 g for 5 min at room temperature. The supernatant was transferred to a new tube and the cell pellets resuspended in 1 mL of fresh phosphate buffered saline (PBS). In order to determine the type of death caused by the compression, an Annexin V/PI [16] assay was carried out and the results analysed using flow cytometry. Three samples were used per group (uncompressed, low pressure and high pressure): they were washed once with PBS, then filtered through a 70-$\mu$m grid and finally resuspended in 100 $\mu$L of Annexin V binding buffer (1x, Invitrogen). FITC-Annexin V (BD Pharmigen) was added at a concentration of 35 ng/mL to each cell suspension followed by incubation at room temperature for 15 min in the dark. Propidium iodide (PI, Invitrogen) was added at a concentration of 10 ng/$\mu$L to each cell suspension followed by incubation at room temperature for 5 min in the dark. All samples were analysed within 30 min using a Becton Dickinson LSR Fortessa flow cytometer.

The content of lactate dehydrogenase in each supernatant was measured to investigate cell lysis and/or cytotoxicity using the LDH-Cytotoxicity Assay Kit II (Abcam) and a Sunrise $^{TM}$ plate reader set to measure absorbance at wavelength 450 nm.

## 3 Results and discussion

### 3.1 Pressure pulses

The pressure developed inside the chamber was assumed to reach a hydrostatic state after an initial ringing period. Previous studies performed on water [11] showed that equilibrium was reached after approximately 20 $\mu$s. In general the duration of each compression pulse was 80 $\mu$s.

In Fig. 1 the average traces obtained from 10 different samples of equal volumes subjected to two different impact velocity conditions (5 samples at 7.0 ± 0.5 m/s and 5 samples at 10 ± 0.5 m/s) are reported. Signals corresponding to the two impact velocities differ both in terms of maximum pressure and impulse. The maximum pressures reached inside the chamber were 11.5 ± 0.5 MPa to 14.2 ± 0.6 MPa and the impulse, calculated by integrating the pressure signal over time for each sample, were 705 ± 30 Pa s and 915 ± 35 Pa s for the low and high impact velocities, respectively. It is still not clear which and how each of these signal components contributes to observed differences in cell viability and morphology, presumably arising from application of compression waves, described below.

### 3.2 Effects on compressed cells

The effects of application of compression waves to MSC samples were initially assessed by comparing cell concentrations of uncompressed and compressed samples. Figure 2 shows a histogram of concentration of cells/mL, averaged over 5 samples from each group (uncompressed, high and low compression) after removal from the sample

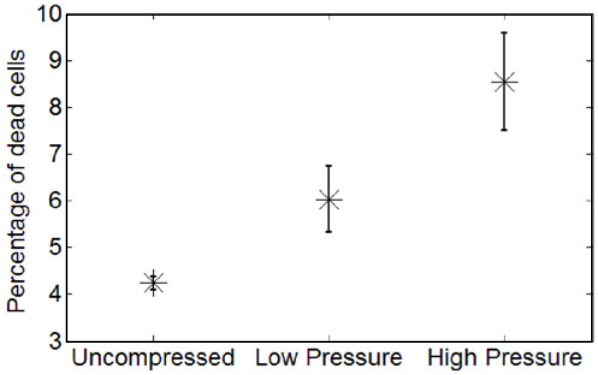


**Fig. 5.** The average percentages of dead cells stained with FITC-Annexin V/PI (PI positive) of uncompressed and compressed (low and high pressure) samples. Five samples form each groups were used, each measured based on counting 10,000 cells per sample.

chamber. No statistically significant difference (by Student's *t*-test) between the initial concentration and the samples in the uncompressed and compressed at low pressure groups were recorded, while a highly significant ($p > 0.01$) decrease in number of cells was recorded for the samples subjected to the higher compressive pulse.

To assess if the decrease in cell concentrations for samples compressed at high pressure was associated with lysis or plasma membrane damage, the amount of lactate dehydrogenase (LDH) in the supernatants was measured. LDH is a stable enzyme usually localised inside cells. When the plasma membrane is ruptured the enzyme can be released into the extracellular medium. Figure 3 shows results obtained from substrate-based assay that correlates the amount of LDH with the production of an assay product that can be measured in $OD_{450\,nm}$ units. A very highly significant ($p > 0.001$, by Student's *t*-test) increase in LDH levels in supernatant was observed in both compressed samples compared to uncompressed samples. Moreover, relative amounts of LDH in the supernatants appears to increase as a function of the pressure pulse. These results suggest that damage due to the pressure pulse may be associated with membrane rupture.

Cell viability in samples subjected to pressure pulses was also assessed using an Annexin V/PI assay. As shown in Fig. 4 most of the cells in all the samples are live cells (Annexin V/PI negative), while a small percentage of them are dead cells (Annexin V/PI positive). Almost no cells are exclusively stained by FITC-Annexin V, which suggests that apoptosis is not induced by the pressure pulses applied to the cells in the samples used here. The average values of the total number of dead cells per each group of samples (10,000 cells per sample) analysed in the FACS assay are shown in Fig. 5 indicating that the relative amounts of dead cells that retain structural integrity increases as a function of the intensity of the applied pulse.

## 4 Conclusions

A system for applying high intensity compression waves to cell cultures has been developed using a modified SHPB setup, equipped with a biocompatible chamber that permits recovery of samples for further cellular and molecular analyses. Altering the impact velocity of the striker bar enables generation of different pressure pulses in liquid samples. Two conditions have been used in these experiments defined as low pressure, corresponding to an impact velocity of $7.0 \pm 0.5$ m/s and at a maximum pressure of $11.5 \pm 0.5$ MPa, and high pressure, corresponding to an impact velocity of $10 \pm 0.5$ m/s and a maximum pressure of $14.2 \pm 0.6$ MPa. Assays of the recovered samples indicate that the system developed is biocompatible since no significant cellular changes were observed for uncompressed samples inserted in the chamber. Significant changes in cell counts, viability and morphologies were observed for the samples subjected to either low or high pressure compression conditions, which suggests that the system is suitable for *in vitro* simulations of blast effects upon biological materials, including living cells. Cellular changes related to damage, studied here, appear to involve a number of processes including lysis and membrane rupture or permeabilisation that not only destroy cells but may also release components that can affect the viability of normal, undamaged cells. In addition, the effects observed appear to increase as a function of the applied pressure.

Research to understand the molecular basis of damage in blast and more generally traumatic injuries is an emerging areas [17]. With appropriate experimental systems that can be used with live cells and other biological materials there is great potential to improve the understanding of how cellular processes can be altered by compression waves and to use this information to develop new therapies to improve clinical outcomes.

## Acknowledgements

This work was generously supported by grants from Atomic Weapons Establishment. The authors would also like to acknowledge Dr Stephen Walley (Cavendish Laboratory, University of Cambridge).

## References

1. Stuhmiller, J. H., Phillips III, Y. Y., and Richmond, D. R., *The physics and mechanisms of primary blast injury* (Conventional Warfare Ballistic, Blast and Burn Injuries)
2. Elsayed, N. M. and Atkins, J. L., *Explosion and Blast-related injuries* (Elsevier Inc, 2008)
3. Zuckerman, S. *Textbook of air armament* (Air Publication, 1952)
4. Zouris, J. M., Walker, G. J., Dye, J., and Galarneau, M.. Military Medicine, **171**, (2006) 246–252
5. Owens, B., Kragh, J. F., Wenke, J. C., Macaitis, J., Wade, C. E., and Holcomb, J. B.. Journal of Orthopaedic Trauma **64**, (2008) 295–299
6. Ramasamy, A., Hill, A. M., Masouros, S. D., Gibb, I., Bull, A. M. J., and Clasper, J. C.. Journal of the Royal Society Interface **8**, (2010) 6890–698

7. Brown, K. A., Bo, C., Masouros, S. D., Ramasamy, A., Newell, N., Bonner, T. J., Balzer, J., Hill, A. M., Clasper, J. C., Bull, A. M. J., and Proud, W. G.. Submitted to APS Proceedings, (2011).
8. Yun, H. C., Branstetter, J. G., and Murray, C. K. Journal of Trauma, **64**, (2008) 163–168
9. Potter, B. K., Forsberg, J. A., Davis, T. A., Evans, K. N., Hawksworth, J. S., Tadaki, D., Brown, T. S., Crane, N. J., Burns, T. C., O'Brien, F. P., and Elster, E. A. Journal of Bone and Joint Surgery, **92**, (2010) 74–89
10. Bo, C., Balzer, J., Brown, K. A., Walley, S. M., and Proud, W. G., European Physics Journal Applied Physics, **55** (2011) 31201:p1–p5
11. Bo, C., Balzer, J., Hahnel, M., Rankin, S. M., Brown, K. A. and Proud, W.G.. Submitted to APS SCCM Conference Proceedings, (2011)
12. Gray III, G. T. *ASM Handbook, Classic split-Hoplinson pressure bar testing*, (ASM Int, 2000).
13. Uccelli, A., Moretta, L., and Pistoia, V. Nature Reviews Immunology, **8**, (2008) 726–736
14. Caplan, A. I. and Bruder, S. P. Trends in Molecular Medicine, **7**, (2001) 259–264
15. Kolf, C. M., Cho, E., and Tuan, R. S. Arthritis Research & Therapy, **9**, (2007) 1–10
16. Wei, H., Li, Z., Hu, S., Chen, X. and Cong, X. Journal of Cellular Biochemistry, **111**, (2010) 967–978
17. Xiao, W. et al. The Journal of Experimental Medicine, **208**, (2011) 2581–2590