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Initiation and Maintenance of Rat Brain Cell Aggregate Culture: A Suitable In Vitro Model for Primary Blast-induced Brain Injury --Manuscript Draft--

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Abstract:	The effect of blast-induced shock waves on brain is unclear. Few clinical cases have been documented where injury has been attributed exclusively to primary blast, and laboratory animal studies are difficult due to scaling issues and the challenges associated with generating primary shock wave insult in isolation from exposure artifacts and other blast components. Tissue culture studies can be used to answer experimental questions as to the direct effects of blast on brain tissue. Rat brain cell aggregate cultures are comprised of multiple differentiated brain cell types that are kept in suspension culture, thus offering a model system where the reflective boundary conditions implicit in surface culture can either be eliminated or controlled/quantitated. A detailed protocol for their initiation and long-term culture is reported in this paper. Results are presented that show that these cultures are sensitive to blast/shock waves in a pressure-dependent manner, and that they can also discriminate between different types of pressure insult. This has mechanistic implications for the study of primary blast-induced traumatic brain injury.
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Thomas W. Sawyer
Casualty Management Section
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3 May 2017

Dear Dr. DSouza,

Please find the revised manuscript with track changes included. I have attempted to answer all of your queries. I have also changed a number of things that I hope will increase the clarity of the protocol. There are two things that I would like to highlight. Based on your first comment on the title, I have submitted a new title that I believe is more appropriate. I will leave it to you to decide as to which title is more suitable for JoVE. In the text I have also designated the Materials/Equipment table as Table 1, with the other table entitled “Suggested Culture Feeding Schedule” as Table 2. I hope that this is consistent with your format. If not, I will need to change the Table designations within the text.

In closing, many thanks for both your time, as well as your patience for this slightly late revised submission.

Sincerely,

Thomas W. Sawyer

TITLE:

Initiation and Maintenance of a Rat Brain Cell Aggregate Culture: A Suitable *In Vitro* Model for Primary Blast-induced Brain Injury

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KEYWORDS:

Blast injury, shock wave, traumatic brain injury (TBI), brain cell aggregates, tissue culture, blast

SHORT ABSTRACT:

The effect of blast-induced shock waves on the brain is unclear due to factors that complicate clinical and experimental data interpretation. Brain cell aggregate cultures can be used to examine the effects of blast waves on biological tissue, without the complication of the boundary effects that impact surface culture studies.

LONG ABSTRACT:

The effect of blast-induced shock waves on the brain is unclear. Few clinical cases have been documented where the injury has been attributed exclusively to the primary blast. Laboratory animal studies are difficult due to scaling issues and the challenges associated with generating primary shock wave insults in isolation from exposure artifacts and other blast components. Tissue culture studies can be used to answer experimental questions relating to the direct effects of a blast on brain tissue. Rat brain cell aggregate cultures are comprised of multiple differentiated brain cell types that are kept in suspension culture, thus offering a model system where the reflective boundary conditions implicit in a surface culture can either be eliminated or controlled/quantitated. A detailed protocol for their initiation and long-term culture is reported in this paper. The results show that these cultures are sensitive to blast/shock waves in a pressure-dependent manner and that they can also discriminate between different types of

pressure insults. This has mechanistic implications for the study of primary blast-induced traumatic brain injury (PbTBI).

INTRODUCTION:

Exposure to a blast can result in injury due to penetrating projectiles or the impact/acceleration effects of being thrown against hard surfaces. While these kinds of injuries are relatively well understood, the effects of blast-induced pressure waves on an individual are much less clear¹⁻³. Although blast waves (primary blast) have been well documented as causing injury to gas-containing organs such as the ear, lungs, and gastrointestinal tract, their effect on solid organs such as the brain is much more controversial^{4,5}. Clinical cases of PbTBI are difficult to diagnose, while the technical challenges associated with generating or simulating a primary blast in the laboratory are significant^{2,3,6}.

Although a variety of animal models of PbTBI have been developed, the unique characteristics of a physical insult, such as a shock wave, make animal models problematic when addressing the biomechanical basis of the potential damage. Thus, while the investigator may desire to ascertain the aspects of a shock wave that are of importance in blast-induced brain damage, the different biomechanical properties of the human versus rat skull adds yet another layer of uncertainty to the already uncertain domain of extrapolating small rodent data to the human condition.

Tissue culture models offer an alternative to whole animals and have the potential to simplify experimental approaches to answer some important questions with respect to the effects of a primary blast on brain tissue. Several laboratories have developed these kinds of model systems, and both primary and established cell lines, as well as brain slice culture models of a primary blast, have been used⁷⁻¹¹. These surface culture model systems use blast and simulated blast exposures generated both underwater and in air. Rat brain cell aggregate cultures are composed of the major cell types of the brain, show many of the same functionalities of the intact brain, and can be cultured for periods of time that extend to months¹²⁻²⁰. They are suspension cultures, and this characteristic allows for the minimization or elimination of complicating boundary conditions during shock wave or blast exposure. This enables additional definition of experimental parameters and also facilitates the measurement of the pressure insults that target cells actually experience. This report gives a detailed protocol to initiate and maintain these cultures. Representative results are also included from previously published blast and simulated blast experiments that incorporated this model system^{15,16}.

PROTOCOL:

In conducting this research, the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation," published by the Canadian Council on Animal Care.

1. Prior to Brain Cell Aggregate Preparation

1.1) Prepare dissection buffer, as described in the **Table of Materials**.

1.2) Prepare culture medium with Basal Medium Eagles (BME) and 5%, 10%, or 15% heat-inactivated fetal calf serum (FCS), as described in **Table of Materials**.

1.3) Prepare culture dishes containing frozen dissection buffer, 6 mL in a 60-mm culture dish and 15 mL in a 100-mm culture dish (one of each culture dish type per preparation).

1.4) Place 10 mL of cold dissection buffer into 100-mm culture dishes (2 per preparation).

1.5) Place ~15 mL of water in 100-mm culture dishes and freeze; these dishes will be used to assist in keeping the tissue cold during brain cell isolation and will be referred to as “cold plates.”

1.6) Place 200- μ m nylon mesh bags (**Table of Materials**) into 100-mL beakers and autoclave.

1.7) Siliconize (**Table of Materials**) spinner flasks and standardize the impeller length. Autoclave.

1.8) Ensure that all materials that will come into contact with the tissue during brain cell preparation or culture are sterilized.

2. Dissection of Embryos from a Pregnant Rat

2.1) Place an anesthetic chamber in a fume or chemical safety hood, or ensure that the volatile anesthetic is scavenged using the appropriate equipment.

2.2) Charge the anesthetic chamber with 3-5% isoflurane/oxygen (2 L/min).

2.3) Place a pregnant rat (17 days of gestation) into the anesthetic chamber until no response is elicited by toe-pinch.

2.4) Quickly perform a cervical dislocation.

2.5) Rinse gloved hands with 70% ethanol.

2.6) Place the animal on its back on an incontinence pad and spray the abdomen with 70% ethanol. Using toothed forceps, grasp a fold of skin at the base of the abdomen and lift.

2.7) Using scissors cut below the skin and fascia, extend the cut up towards the sternum and expose the peritoneal cavity.

2.8) Using scissors and forceps, excise the uterus containing the fetuses into a 100-mm Petri dish containing cold dissection buffer.

2.9) Quickly transport the dish of embryos to a laminar flow hood and place it onto a cold plate.

2.10) Wrap the rat carcass in the incontinence pad, place it in a biohazard bag, and dispose of the waste as per institutional procedures.

3. Dissection of Brain Tissue and the Initiation of Brain Cell Aggregate Cultures

3.1) Isolate the embryos immediately upon receipt. Grasp the uterus with forceps and, using small curved scissors, place the embryos in a 100-mm culture dish containing cold dissection buffer. Count and record the embryo number.

3.2) Break up the frozen dissection buffer in a 100-mm culture dish. Place it on a cold plate.

3.3) Isolate the brains by gently holding the neck of an embryo using curved forceps. Using fine curved scissors, make a shallow lateral cut between the eyes and extend it half way up towards the top of the head. Extract the brain by gently squeezing the top of the skull between the scissor blades or by using curved forceps.

3.4) Place the brains from the embryos collected from one animal into the thawed portion of the dissection buffer in the 100-mm Petri dish.

3.5) Break up the frozen dissection buffer in a 60-mm Petri dish. Place it on a cold plate.

3.6) Using two pairs of fine forceps, dissect out the forebrains and remove the meninges. Place them in the thawed portion of the dissection buffer in the 60-mm dish; this can be done by eye but may require a dissecting microscope or surgical loupes.

3.7) Transfer the forebrains and dissection buffer into 5- mL centrifuge tubes and place them on ice.

3.8) Rinse the forebrains three times with 35-40 mL of cold 10% FCS BME, discarding the supernatants (the forebrains will quickly sink to the bottom).

3.9) Adjust the volume to ~5.0-7.5 mL with cold 10% FCS BME after the last rinse and gently triturate eight times with a Pasteur pipette.

3.10) Adjust the cell suspension volume to ~35 mL with 10% FCS BME and let unsegregated tissue settle to the bottom of the 50-mL centrifuge tube for ~1-2 min.

3.11) Carefully pour the suspension through 200- μ m nylon mesh into the 100-mL beaker, leaving 3-5 mL of the bottom portion containing the unsegregated tissue.

3.12) Pour the filtrate into a 50-mL centrifuge tube and place it on ice.

3.13) Take the unsegregated tissue from step 3.11 and again gently triturate eight times with a Pasteur pipette. Filter the suspension through the same nylon mesh and then rinse the mesh with ~15 mL of cold 10% FCS BME. Consolidate all filtrates in the same 50-mL centrifuge tube.

3.14) Centrifuge for 15 min at 300 x g and 4 °C.

3.15) Carefully decant off the supernatant and suspend the cell pellet in ~10 mL of cold 10% FCS BME using gentle trituration (3-5 times) with a 10-mL pipette. Make up to ~50 mL with cold 10% FCS BME.

3.15.1) If the cells are to be counted, take a 100-μL sample and dilute up to 10 mL in 10% FCS BME in a 15-mL centrifuge tube. Count the birefringent cells on a hemocytometer.

3.16) Centrifuge the 50-mL centrifuge tube for 15 min at 300 x g and 4 °C.

3.17) Decant off the supernatant and gently suspend the cells in ~10 mL of cold 15% FCS BME using a 10-mL pipette.

3.18) Seed a siliconized spinner flask so that the cells from the embryos of one pregnant animal are placed into a final total volume of 80 mL of cold 15% FCS BME.

4. Culture and Maintenance of Rat Brain Cortical Aggregates

Note: See Table 1.

4.1) Immediately center the spinner flask on a magnetic stirrer set at a rotational velocity of 60 rpm. Place it in a humidified 7% CO₂ incubator at 37 °C.

Note: It is important to monitor the CO₂ levels and temperature in the incubator to ensure optimal culture viability.

4.2) Check the outside of the flask for condensation within the first 10 min and wipe it off.

Note: Failure to do so will often result in the flask moving slightly and can cause the impeller to impact the side of the spinner flask. This will substantially reduce viability.

4.3) Add 20 mL of pre-warmed (37 °C) 5% FCS BME to the spinner flask after 2 h and adjust the rotational velocity to 70 rpm.

4.4) At 24 h, add 25 mL of pre-warmed (37 °C) 5% FCS BME to the spinner flask and adjust the rotational velocity to 75-78 rpm. Keep this 125-mL volume constant for the remainder of the culture life.

4.5) At two days, adjust the rotational velocity to 78-80 rpm.

4.6) At three days, take a ~1.0-mL sample and place it into one well of a 24-well multiwell plate for visual inspection under the microscope (see the **Table of Materials**, 100x magnification); the aggregates should be ~80-120 μm in size, with single cells in the background. Increase the rotational velocity to 80-82 rpm.

4.6.1) Slightly decrease (by 2-3 rpm) the suggested (80-82 rpm) rotational velocity of the impeller if the aggregates are too small, or slightly increase (by 2-3 rpm) it if they are too large.

4.7) On days 6, 8, and 10, feed the aggregates with 60 mL of 10% FCS BME and increase the rotational velocity to 85-87 rpm; the aggregates should predominantly be spherical and ~200-400 μm in size.

4.7.1) Slightly decrease (by 2-3 rpm) the suggested (85-87 rpm) rotational velocity of the impeller if the aggregates are too small, or slightly increase (by 2-3 rpm) it if they are too large.

4.8) Feed the cultures with 35 mL of 10% FCS BME on day 12.

Note: This step is required only if the cultures are judged to be sufficiently metabolically active that they will acidify the medium to unacceptable levels over a three-day interval. This can be determined through the assessment of phenol red-indicated medium acidity or medium pH determination.

4.9) Feed the cultures with 90 mL of 10% FCS BME starting on day 13; between ~13 and 20 days, they are metabolically very active. If the cultures acidify the medium to unacceptable levels (*i.e.*, the medium turns yellow) between the ~2-day feeding intervals (see **Table 1**), split the cultures so as to satisfy nutrient requirements and maintain the pH at ~7.4.

Note: The background should be clear and the aggregates angular in shape, with irregular edges.

4.9.1) To split the cultures, allow the aggregates to sediment to the bottom of the spinner flask, gently aspirate ~50% of them into a 35-mL pipette, and seed a new spinner flask. Split the old medium evenly between the two flasks and make up to 125 mL with fresh 10% FCS BME.

4.10) Feed the cultures with 90 mL of FCS BME on days ~20-27; the cultures become spherical in shape again, are ~300-450 μm in size and have a clear background.

4.11) Use the mature cultures for experimental purposes on days ~27-29. Feed the cultures with 50-60 mL of 10% FCS BME after treatments.

5. Exposure of Aggregates to Primary Blast and Shock

Note: See^{15,16} for complete details on exposure conditions and methodologies.

5.1) On the day of experimentation, isolate the aggregates from the spinner flasks by sedimentation and combine. Retain the conditioned medium for use with the aggregates after shock/blast exposure.

5.2) Using a 5-mL pipette, perform gentle trituration of the combined aggregates. Divide the aggregates equally into the desired number of test samples in 50-mL centrifuge tubes.

Note: The total aggregate cellular material required for each test sample (usually measured as protein) will depend on the experimental endpoint assay requirements. This will vary according to the experimental protocol.

5.3) Place the aggregates (suspended in conditioned 10% FCS BME) into dialysis tubing (**Table of Materials**) for underwater explosion (UNDEX) exposure or into spheres (**Table of Materials**) for simulated air blast exposure.

Note: The sham controls used during these studies consist of aggregates placed into either dialysis tubing or into spheres. These samples are prepared and treated in an identical manner as the test samples, except for the exposure to a blast or simulated blast.

5.4) Expose the aggregates to a blast/shock^{15,16}, ensuring that the aggregates are in suspension by inverting the dialysis tubing just prior to the UNDEX explosion or by turning the sphere 180° just before an advanced blast simulator (ABS)-generated air blast.

Note: Exposure conditions will vary according to the laboratory capabilities and test platforms. The studies conducted here and used as examples in this report used single-pulse UNDEX exposures of ~43-2,071 psi (0.1-0.4-ms duration)¹⁵ and single-pulse simulated blasts of 15-30 psi (~6.4-7.7-ms duration)¹⁶.

5.5) Place the aggregates back into routine culture conditions after exposure, using equal volumes of conditioned medium and fresh 10% FCS BME supplemented with antibiotics, as identified in the **Table of Materials**.

Note: The aggregates are outside of routine culture conditions for ~20-30 min, which is controlled for by using sham controls that experience the same manipulations but without blast exposure.

REPRESENTATIVE RESULTS:

The cells are seeded into 125-mL spinner flasks that are placed onto four-place magnetic stirrers (**Figure 1A**) located in a humidified 37 °C incubator set at 7% CO₂. Initially, the medium is opaque in appearance, but by ~6 days, the medium clears and individual aggregates can be discerned with the naked eye (**Figure 1B**). Initially, the aggregates have irregular edges, but with time in culture, the outer edge becomes smooth as seen under a light microscope (**Figure 2A**). Electron microscopy shows the detail of the multicellular aggregates (**Figure 2B**). The brain cell

aggregates are comprised of multiple cell types, including microglia and oligodendrocytes (**Figure 2C**), as well as glial and neuronal cells that organize themselves into a defined and characteristic architecture with time (**Figure 2D**).

Exposure of brain cell aggregates to blast and shock can be accomplished through a variety of platforms and methodologies, depending on the expertise and capabilities of the test laboratory. DRDC Suffield Research Centre facilities include an UNDEX pond (**Figure 3A**), as well as an ABS (**Figure 4A**). The examples included below are derived from previously published work using these facilities^{15,16} and can be used as guidelines for studies using other types of facilities.

Aggregate exposure to an underwater blast was obtained by encasing mature aggregates in dialysis tubing (**Figure 3B**) and lowering them into an UNDEX pond (**Figure 3A**) prior to the detonation of C4 charges. The dialysis tubing is transparent to blast waves, while the large size of the pond prevents reflections, thus exposing the aggregates to highly defined single-pulse blast waves. **Figure 3C** shows that the blast wave profiles were virtually identical whether they were measured just outside or inside the dialysis tubing (**Figure 3B**). Exposure of the aggregates to much more complex shock wave-induced pressure changes was accomplished by loading the aggregates into polypropylene spheres that were screwed onto a support assembly using a bolt (**Figure 4B**). This assembly was then positioned into the ABS (**Figure 4A**) across from a pressure gauge to measure static overpressure. Although the 25-psi simulated air blast exposure of the sphere was single-pulse in nature, the actual pressure changes (as measured by piezoresistive sensors²²) experienced by the aggregates inside the sphere were complex in nature (**Figure 4C**).

Exposure of aggregates to both underwater blasts (**Figure 5A**) and simulated air blasts (**Figure 5B**) resulted in similar effects on the cellular survival of protein Akt after 3 days. While the total amount of Akt was unaffected, both types of exposure resulted in a significant and pressure-dependent elevation of phosphorylated Akt (p-Akt). At longer time points, this elevation returned to control values (data not shown). The underwater blast caused vascular endothelial growth factor (VEGF) levels to decrease after 7 days, which then rebounded towards control values after 28 days (**Figure 6A**). In contrast, the air blast caused a significantly delayed and pressure-dependent decrease in VEGF levels only after 28 days (**Figure 6B**). All exposed samples are compared to sham controls that underwent the same exposure procedures but without a blast or simulated blast.

FIGURE AND TABLE LEGENDS:

Figure 1. Spinner flasks placed on magnetic stirrers. A) Rat brain cells are placed into spinner flasks, and the cells are kept in suspension using impellers set at varying rotational velocities, depending on the stage of the culture. B) Initially, the cell suspensions are opaque, but as they aggregate and grow in size, the aggregates become visible to the eye (day-22 aggregates are depicted).

Figure 2. Representative micrographs of rat brain aggregates. A) Light micrograph of 28-day-old aggregates. B) Electron micrograph of a 25-day-old aggregate showing cellular topography. C) Confocal image of a 27-day-old aggregate with 4'6-diamidine-2' phenylindole (DAPI)-stained nuclei (blue; Exc: 405 nm/Em: 461 nm), oligodendrocyte (red; Exc: 561 nm/Em: 647 nm; myelin basic protein), and microglial (green; Exc: 488 nm/Em: 546 nm; Iba1) cell populations. D) Confocal image of a 25-day-old aggregate showing typical architecture, with an outer glial (green; Exc: 488 nm/Em: 546 nm; glial fibrillary acidic protein) coating surrounding a largely neuronal (red; 561 nm/Em: 647 nm; NeuN) interior.

Figure 3. UNDEX exposures. A) The UNDEX pond has a diameter of 50 m and a maximum depth of 7 m at the center. It is shaped as an inverted, truncated cone and holds over 7,000,000 L of fresh water. B) The aggregates are loaded into ~10 cm-long cylinders of dialysis tubing secured on either end with surgical thread, and the sausage is clipped to a fishing line before being lowered into the water. C) The traces represent pressure readings taken just outside (red) or inside (blue) the dialysis tubing cylinder. The figure is a modification of Figure 4B in¹⁵.

Figure 4. ABS exposures. A) The ABS uses a divergent driver (left-hand side) and an end-wave eliminator (right-hand side) to simulate single-pulse blast waves with high fidelity. B) The aggregates are loaded into spheres that are screwed onto a support assembly using a threaded bolt, and the assembly is positioned in the ABS opposite a pressure gauge to measure static overpressure. C) Although the 25-psi (~7.7-ms duration) simulated air blast exposure of the sphere is single-pulse, the actual pressure changes experienced by aggregates inside the sphere are complex in nature. The trace depicted is of pressure changes measured at the center of the sphere. The figure is a modification of Figure 3B in¹⁶.

Figure 5. Effect of primary blast on brain aggregate Akt levels. Brain cell aggregates were exposed to A) low- (~43 psi), medium- (~397 psi), or high- (~2,070 psi) pressure underwater blasts or to B) 15-, 20-, 25-, or 30-psi primary air blasts in the ABS and then placed back into culture. Cultures were assessed for changes in total or phosphorylated Akt (p-Akt) levels using Western blotting at 3 days post-exposure. No differences between sham control and blast-exposed aggregates were observed in the total Akt. However, the primary blast caused significant and pressure-related increases in p-Akt levels at 3 days post-exposure using both exposure regimens. Data were analyzed using two-way ANOVA and a Dunnett's test (asterisks denote statistical differences from sham control values, $p < 0.05$, $n = 3$). Sham control samples were treated identically to exposed the samples, but without exposure to a blast or simulated blast. =This figure is derived from previously published data^{15,16}.

Figure 6. Effect of a primary blast on brain aggregate VEGF levels. Brain cell aggregates were exposed to A) low- (~43 psi), medium- (~397 psi), or high- (~2,070 psi) pressure underwater blasts or to B) 15-, 20-, 25-, or 30-psi primary air blasts in the ABS and then placed back into culture. Cultures were assessed for changes in VEGF levels using an immunoassay at 7 and 28 days post-exposure. The maximal VEGF decline was observed 7 days after exposure to the underwater blast, in contrast to VEGF expression in aggregates exposed to the air blast, where inhibition was delayed to 28 days. The data were analyzed using two-way ANOVA and a

Dunnett's test (asterisks denote statistical differences from sham control values, $p < 0.05$, $\bar{x} \pm \text{SEM}$, $n = 3$). Sham control samples were treated identically to exposed samples, but without exposure to a blast or simulated blast. This figure is derived from previously published data^{15,16}.

Table 1. Suggested culture feeding schedule.

DISCUSSION:

The successful preparation and maintenance of brain cell aggregate cultures requires vigilance and attention to detail. During culture initiation, it is important to work quickly and efficiently. Brain tissue should be kept as cold as possible but should not come into direct contact with ice. The seeding of the cultures is not predicated on cell seeding number, but rather on the embryos from one animal. Typically, 9-12 embryos/dam are collected, with viable (birefringent) cell yields ranging between 20-24 million cells/embryo for the cell preparation used to seed one spinner flask. Cell counts and viability assessments (*i.e.*, using trypan blue) are used primarily as quality-control guidelines, with viabilities ranging between ~36 and 41%. The variability in seeding density is subsequently addressed by altering the feeding volumes and schedules and the rotational velocities of the spinner flask impellers, as well as by sub-culturing the cultures if nutrient requirements become greater than can be accommodated using 3-4 medium changes per week. It is recommended that the cultures are checked visually (to detect aggregate clumping) and microscopically at least every two days. At all times during culture, it is critical to ensure that the spinner flask is properly centered on the stirrer to prevent the impeller from hitting the sides. The impacts resulting from improperly placed spinner flasks will rapidly destroy the culture. Therefore, it is recommended that they be checked at least twice after each manipulation to ensure that they remain properly centered. Cultures that are damaged at any time should be discarded and not used for experimentation.

Aggregate size is determined by the initial cell populations seeded, as well as by the sheer produced by the impellers, which is dependent on their length, their rotational velocity, and the geometry of the spinner flasks. Minute changes in these variables, of which only rotational velocity can be closely controlled, can result in cultures whose growth will vary, even between flasks seeded with aliquots of the same cell suspension. During the first ten days in culture, the rotational speed can be slightly reduced in those cultures whose aggregates are judged to be too small for that stage of the culture. In contrast, if the aggregates are judged to be too large, the speed can be slightly increased. It should be noted that the protocol instructions for aggregate size and rotational speed, as well as those outlined in **Table 1** for refeeding the cultures, have been derived specifically from studies using the media, stirrers, and spinner flasks defined in this protocol. Materials used from other sources will change these guidelines.

Rat brain cell aggregate culture has been well characterized as a powerful model system with which to examine the pathophysiology of disease processes and xenobiotics in the central nervous system^{12-14,17-20}. They are comprised of the major cell types of the brain, including neurons, astrocytes, microglia, and oligodendrocytes (but not endothelial cells or fibroblasts)^{12,13}, and that, in mature aggregates, are found in similar ratios compared to the human cortex or rat brain¹². The multicellular aggregates are dynamic in nature, growing and

differentiating with time in culture until they exhibit many of the functions found in the intact brain. While this maturation process occurs slowly, resulting in studies of several weeks in duration, their suspension nature especially lends itself to studies involving blasts and shock. Although surface cultures may be used in these kinds of studies, the presence of culture surfaces and culture vessel walls act as boundary conditions, causing complex reflections and rendering the measurement of the actual pressure insult difficult, if not impossible. Furthermore, the presence of a support surface with different mechanical properties below and adhered to either the monolayer or explant/slice cultures may change the biomechanical properties of the tissue being studied, further complicating the assessment of cause-effect relationships.

In the experimental examples used in this protocol, the exposure conditions experienced by the aggregates in response to underwater blasts and simulated air blasts represented two very different kinds of insult. In the underwater blast exposures, the large size of the UNDEX pond minimized reflections from the sides of the pond, while the dialysis tubing itself was transparent to the blast wave. The exposure experienced by the aggregates thus consisted of a well-defined, single-pulse shockwave¹⁵. In contrast, the aggregates encased within a spherical shell and exposed to a single-pulse air shockwave experienced complex and spatially dependent pressure changes due to the reflecting boundaries of the interior of the sphere^{16,22}. In both cases, the effect of the blast or simulated blast were subtle, and many of the biomarkers commonly used as measures of traumatic brain injury *in vivo* (*i.e.*, GFAP; neurofilament, inflammatory, and enzymatic endpoints; or cell death (LDH, caspase-3)) were not changed^{15,16}. This is consistent with other *in vitro* investigations of primary blasts⁷⁻¹¹, as well as with the notion that a primary blast may induce a type of TBI distinct from that caused by impact or acceleration/deceleration. However, the aggregate cultures did respond to pressure insults, with changes in the cellular survival proteins Akt and VEGF. In both types of exposures, p-Akt was elevated at early time points and then declined. In contrast, the effects on VEGF levels were dependent on the type of exposure. This protein declined at early time points when the aggregates were exposed to single-pulse underwater blasts but declined only in a much delayed fashion 28 days after the air blast exposures^{15,16}. VEGF has been implicated in several neurodegenerative diseases, and its delayed inhibition by air blast is noteworthy.

Rat brain cortical cell aggregate cultures are a powerful tool with which to study the effects of blasts and shock on brain tissue. They are sensitive to pressure changes and can be used for biomechanical, mechanistic, and protective drug studies. In addition, their ability to discriminate between different kinds of pressure insults illustrates their potential value in elucidating which aspects of pressure change are of importance in determining the biomechanical basis of PbTBI. In this protocol, two examples^{15,16} are used to illustrate the utility of the brain cell aggregate model system in blast and shock studies. The UNDEX facilities are unique, while the design of the ABS is proprietary; thus, a detailed description of these studies is not included. However, the unique characteristics of the brain cell aggregates, as well as the ability to place them into suspension in vessels with different types of geometries/mechanical properties just prior to exposure, can be taken advantage of in other laboratories using other types of exposure platforms.

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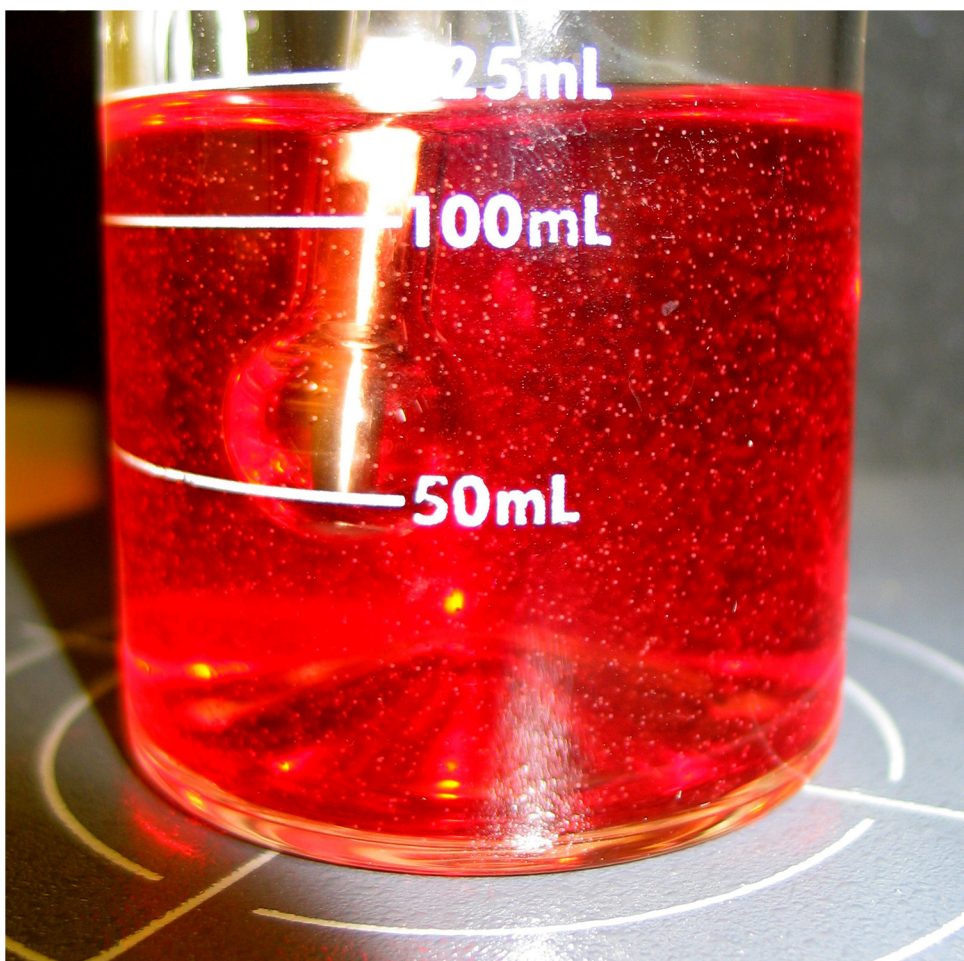
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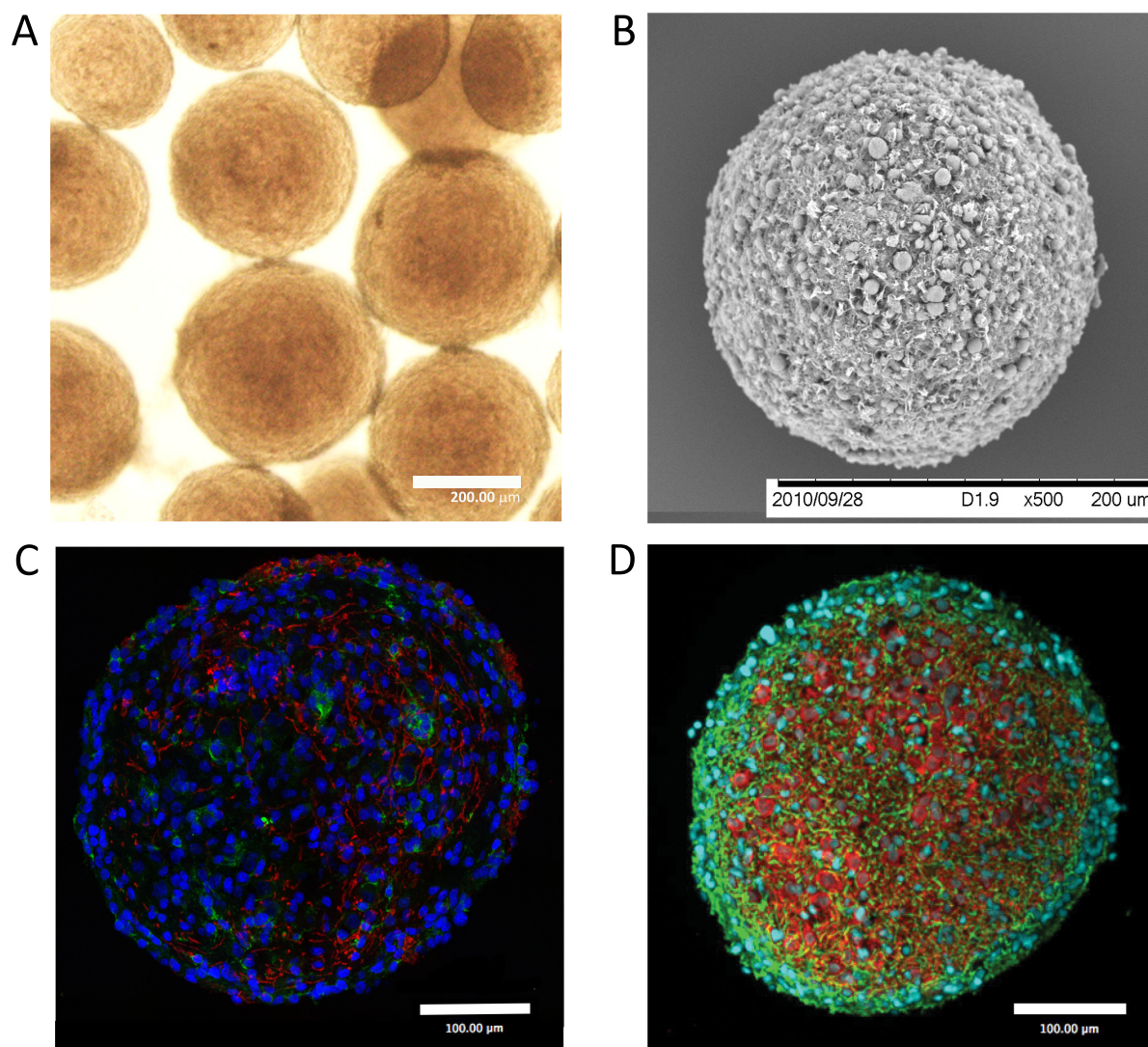
The authors have nothing to disclose.

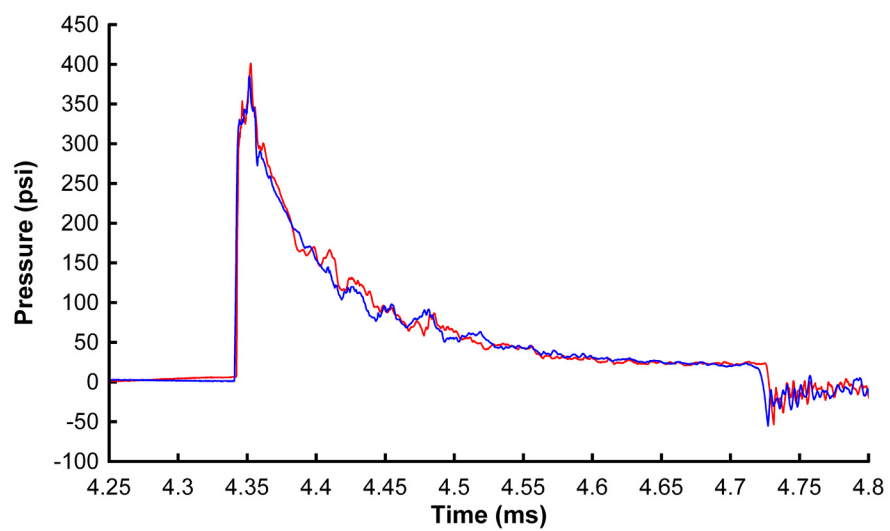
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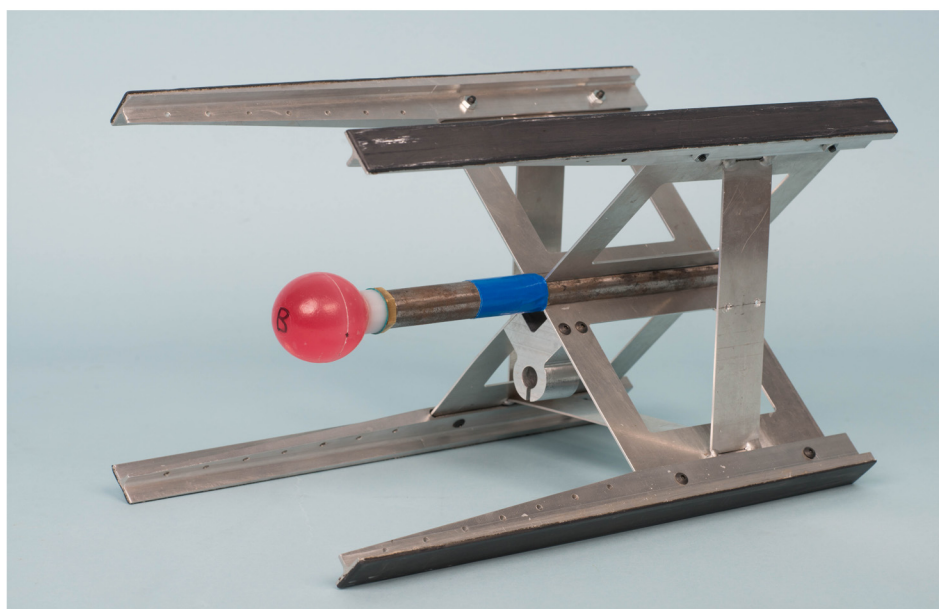
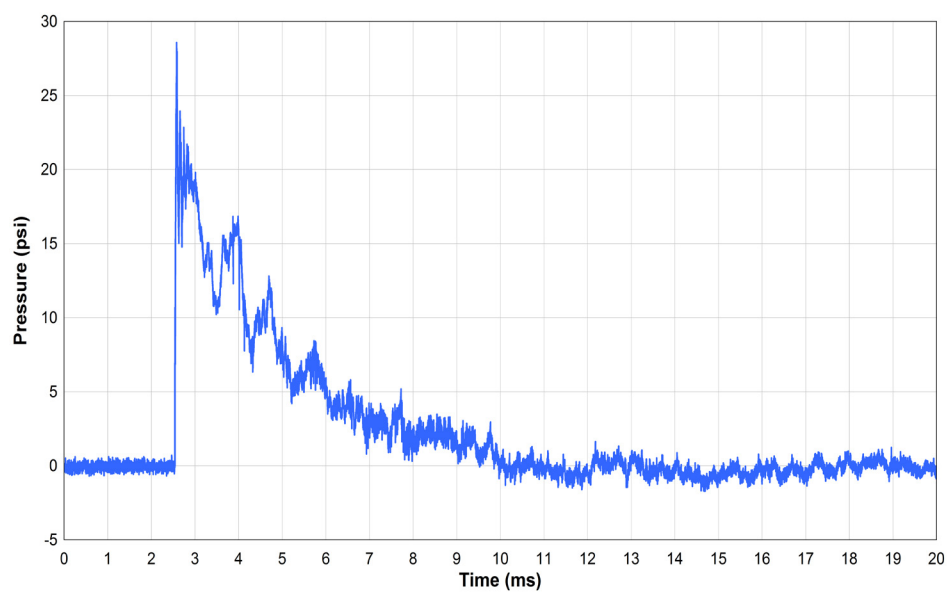
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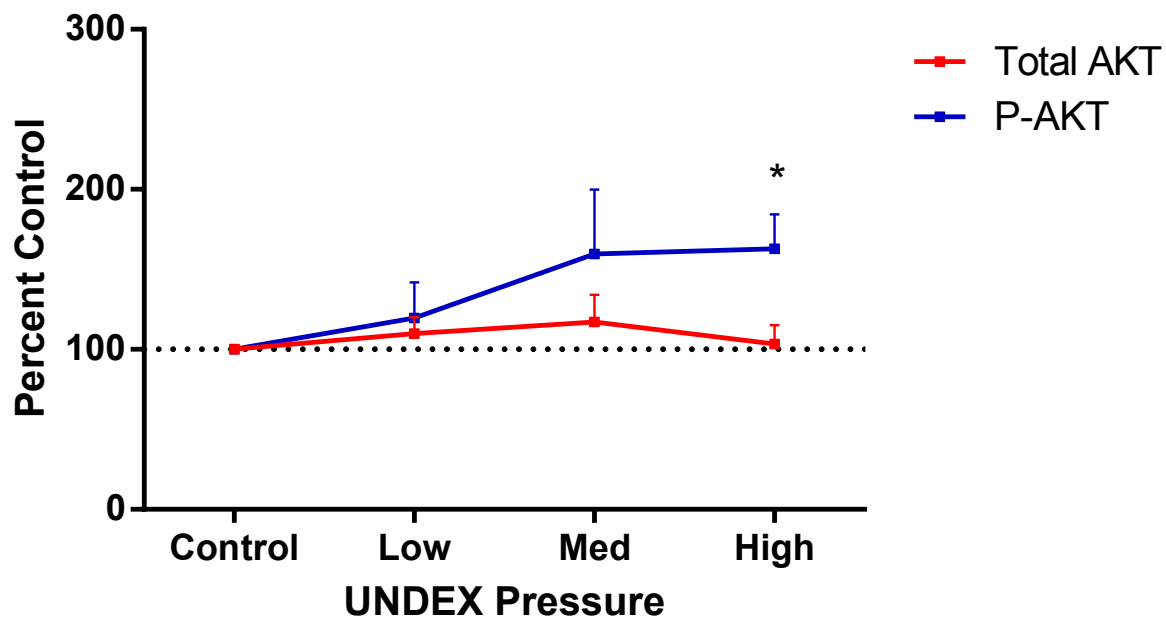
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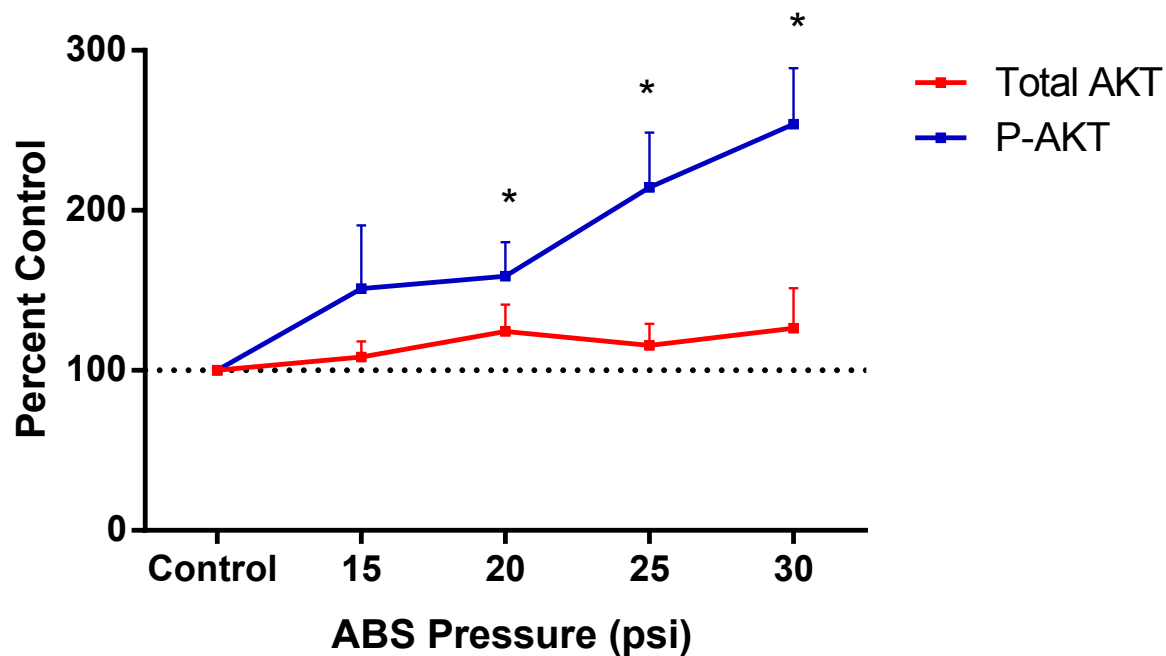
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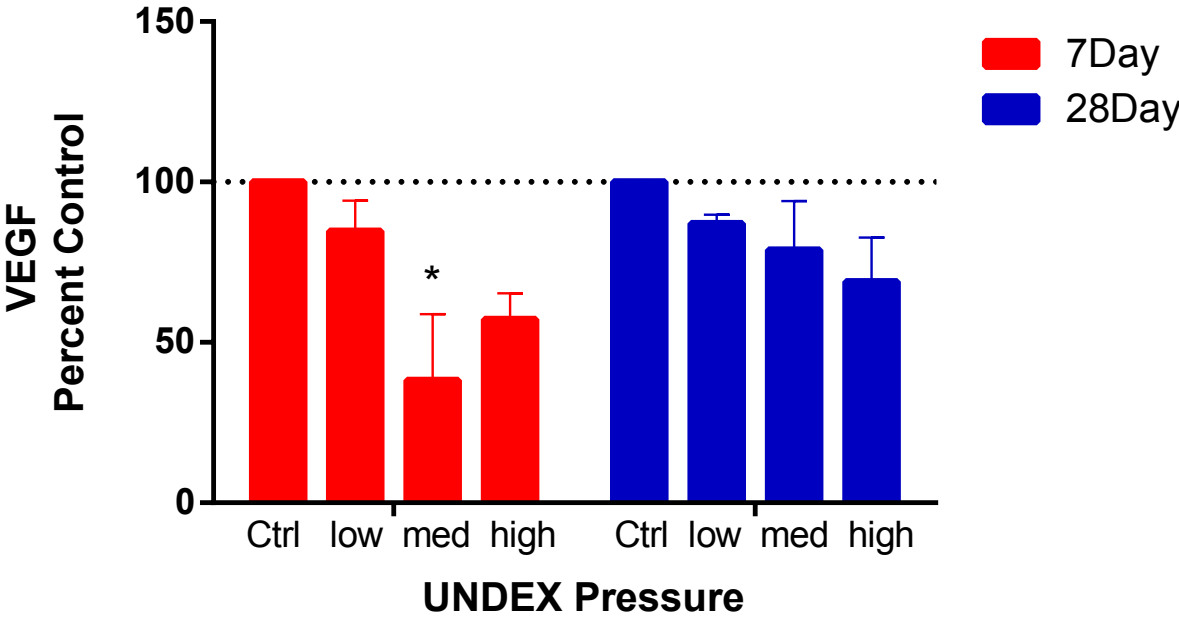
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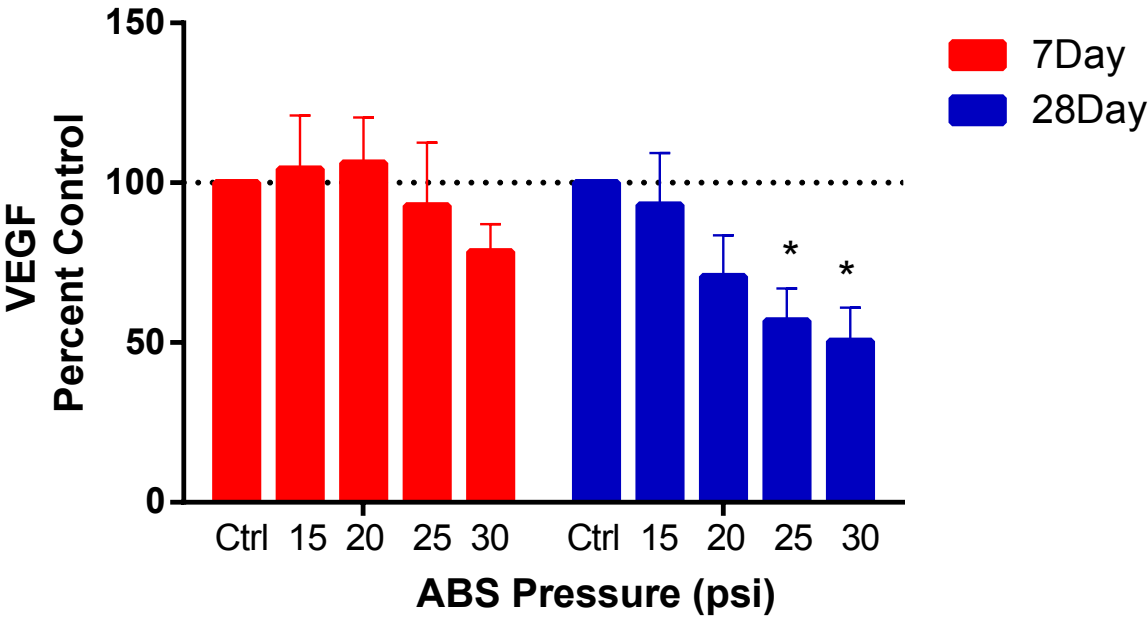
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Days <i>in Vitro</i> after initiation <i>Tuesday = Day 0</i>	10% FCS BME Feeding volume (mL)
Day 1 – 5 (Wed – Sun)	no medium change
Day 6, 8, 10 (Mon, Wed, Fri)	60
Day 12 (Sun)	35 [#]
Day 13, 15, 17 (Mon, Wed, Fri)	90
Day 19 (Sun)	35 [#]
Day 20, 22, 24 (Mon, Wed, Fri)	90
Day 27 (Mon)	90
Post-treatment (Mon, Wed, Fri)	50-60

* Guidelines only

If required

Material/Equipment	Company Catalogue #	Comments
Hanks Balanced Salt Solution (HBSS)	Various	
Dissection buffer	Prepared in-house	15 mM HEPES, 27.8 mM glucose, 58.4
Basal Medium Eagle (BME liquid)	Sigma-Aldrich Cat# B1522 (500 mL bottle)	Can be purchased either as liquid, or as a powder Other suppliers are available
Basal Medium Eagle (BME powder)	Sigma-Aldrich Cat# B9638-10X1L	
US Qualified heat inactivated foetal calf serum	Life Technologies Cat# 16140071	Other suppliers available
Culture media (5-15% FCS BME)	Prepared in-house	BME supplemented to 5-15% FCS, 2 mM L-
SigmaCote	Sigma-Aldrich Cat# SL2-100mL	Used to coat spinner flasks as per
Nitex nylon mesh (200 µm)	Sefar Inc. 111 Calumet St. Depew, New York USA 14043	The nylon mesh bags are custom ordered and made to fit a 100 mL beaker. The bags
Scissors	Various	Sterile
Large toothed forceps	Various	Sterile
Anaesthetic system	E-Z Anesthesia P.O. Box 3544 Palmer, PA USA 18043 EZ-7000 Classic System	Other suppliers available
Isoflurane	Baxter Corp. Mississauga, ON Canada	Other suppliers available
Sprague-Dawley rats Timed pregnant	Charles-River St. Constant, QC Canada	Other suppliers available
Incontinence pads	Various	
60 and 100 mm disposable culture dishes	Various	Sterile
Biohazard bags	Various	
70% ethanol spray bottle	Various	Used throughout the procedures

Gloves, masks	Various	
Haemocytometer	Various	
Refrigerated centrifuge	Various	Set at 300 x g, 4 °C
Curved scissors (small)	Various	Sterile
Curved forceps (small)	Various	Sterile
Ice bucket	Various	
Glass rod	Various	Sterile
Pasteur pipettes	Various	Sterile
100 and 500 mL beakers	Various	Sterile
15 and 50 mL disposable centrifuge tubes	Various	Sterile
Microscope	Various	
Laminar flow hood	Various	
Humidified CO ₂ incubator	Various	Set at 7% CO ₂ and 37 °C
10 and 25 ml disposable pipettes	Various	Sterile
Magna Flex 125 mL Spinner Flasks	VWR Cat# 62402-240	With adjustable impellers.
Variomag Biosystem 4 Direct Stirrer	Fisher Scientific Cat# 04979234	Stirrers from different sources may
Variomag Biomod 40B 115V	Fisher Scientific Cat# FS5P97 08789	
24 well multi-well culture dishes	Various	For routine microscopic assessment of aggregates
Dialysis tubing	BioDesign Inc. of New York PO Box 1050 Carmel, New York, USA 10512 Cat# D106	“BioDesignDialysis Tubing” (100 foot roll, 1.13 inch wet diameter, 6.45 mL/cm volume). 8000 Dalton cut-off Sterilized and impermeable to bacteria.

Hollow polypropylene balls	Precision Plastic Ball Co.	Spheres custom prepared for placement into Advanced Blast Simulator (ABS)
2 inch diameter	10129 Pacific Ave Franklin, IL, USA 60131 Cat # 25002.000MPP	~1 mm wall thickness
Piezoresistive sensors	Millar SPR-407	
Microscope	Zeiss Axiovert 40 CFL	
NeuN antibody	Millipore Cat# MAB377	Dilution = 1:200
Glial Fibrillary Acidic Protein (GFAP) antibody	EMD Millipore Corp Cat# AB5804	Dilution = 1:500
Iba1 antibody	Wako Chemicals USA Richmond, VA, USA Cat# 019-19741	Dilution = 1:500
Myelin Basic Protein (MBP) antibody	EMD Millipore Corp Cat# MAB382	Dilution = 1:50
Phosphorylated Akt antibody	Cell Signaling Technology MA, USA Cat# 9271	Dilution = 1:500
Akt antibody	Cell Signaling Technology Cat# 9272	Dilution = 1:1000

* All materials coming into contact with the brain tissue to be cultured, or in culture

Methods Section #
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Thomas W. Sawyer
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3 May 2017

Dear Dr. DSouza,

I have included the answers to all queries using track changes in the manuscript. I hope that this is acceptable.

Sincerely,

Thomas W. Sawyer