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Untitled Eckardt Thesis by A. Sidwell

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability in persons under 35 years of age. In nonfatal moderate to severe TBI, secondary cellular edema causes long-lasting brain damage. Vasopressin mediates the expression of aquaporin 4 after injury, a protein that regulates water uptake into cells and thus contributes to brain swelling. I examined the role of vasopressin in aquaporin 4 upregulation in chemical and mechanical models of TBI. Vasopressin was used to simulate TBI conditions in culture and upregulated aquaporin 4 following injury condition. A blast device was created to simulate blast injury conditions \textit{in vitro}. Future experiments are needed to refine device parameters and its potential use as a high throughput screening mechanism for drugs that can potentially treat TBI.

Background

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in individuals under the age of 35 in the United States, contributing to a third of all injury-related deaths. There are an estimated 1.7 million cases of TBI each year in this country, 1.4 million of which are treated in emergency rooms and 52,000 result in death. Military personnel sustain over 30,000 medically diagnosed cases of TBI annually. In total, these injuries amount to an annual economic burden estimated at $76.5 billion (1, 2).
Traumatic brain injuries come from concussive or penetrating injuries. Motor vehicle accidents, sports concussions, and accidental falls involving head trauma are crash injuries, meaning that the brain collides with the inside of the cranium, ricochets, and strikes the opposite surface, causing bilateral damage. Blast injuries are more common in combat or certain industrial settings and result from exposure to a blast wave. The shockwave propagates through the fluid in the brain, accelerating and damaging the tissue (3). TBI are categorized as mild, moderate, or severe based on the intensity of symptoms. Motor control, emotional regulation, and cognitive function are disrupted by TBI. If the injury itself is not fatal, the secondary swelling that occurs in brain cells can cause long-lasting or permanent damage. Brain volume increases due to water uptake, which leads to elevated intracranial pressure (4).

Arginine vasopressin (AVP) is a peptide neurotransmitter hormone produced in the hypothalamus. Descending projections from the sites of synthesis, the supraoptic and paraventricular nuclei, transport AVP to the posterior pituitary, where it is released into the peripheral circulation. Ascending projections from these nuclei are found throughout the limbic system and cortex, where AVP release is involved in the regulation of numerous social behaviors, brain water permeability and cerebral vascular resistance after severe injury.

AVP contributes to the pathophysiology of brain edema following injury through its effects on AQP4. After a TBI, AVP is released centrally and the transient disruption of the blood-brain barrier simultaneously allows AVP from the peripheral circulation to enter the brain. This normally would not occur because AVP does not cross the blood brain barrier. The flood of AVP is advantageous to constrict blood vessels after a laceration, but detrimental in the brain where its presumed effect on AQP4 increases cellular water retention and pressure.
There are three AVP receptor subtypes: V1a and V1b, which are expressed in the brain, and the V2 receptor, expressed in the kidney. The AVP V1a receptor (V1aR) subtype is densely expressed in cortical and subcortical brain areas across all mammals, including humans. The magnitude of AVP increase following TBI has been reported in several studies to correlate with negative outcomes, strongly suggesting that AVP plays a role in the development of brain damage following concussive injury. In support of this notion are multiple studies in rats showing increased AVP expression in the hypothalamus and cortex and elevated AVP V1aR levels in astrocytes and cerebrovascular endothelium after experimental TBI (5, 14, 15). The change in AVP expression and the V1a receptor occur between 4 and 24 hours post trauma. Other documented biomarkers following TBI include changes in calcium, aquaporin 4, S100B, glial fibrillary acidic protein, neuron specific enolase, and ubiquitin carboxyl-terminal hydrolase L1 (8-11). Biomarkers are used to assess severity of injury and develop treatment plans. They can also be used in vitro to study the signaling cascade in the brain following injury.

Previous literature suggested the efficacy of vasopressin V1a receptor antagonists in mediating the deleterious effects of TBI, specifically through its interaction with aquaporin 4 (AQP4), a water channel protein (5). The AQP4 channel is central to the influx of water and resulting edema in the post-injury signaling cascade. AQP4 expression is associated with high mortality and morbidity in patients with TBI (12, 13). Marmarou et al. (4) found that a selective V1a antagonist, SR49059, could prevent brain edema, reduce swelling of astrocytes, and reduce TBI biomarker expression including V1aR and AQP4 after focal injury (4). However, the V1aR antagonist was infused immediately after injury for five consecutive hours. Kleindienst et al. (5) also found that a V1aR antagonist downregulated AQP4 expression, but the antagonist was administered prior to injury. This antagonist is selective for the V1aR.
TBI can be mimicked in vitro using the addition of AVP to astrocytes or primary neuronal culture. Brain slices, organotypic cultures, cell lines, and dissociated primary cultures can each be used in in vitro TBI models (16). Blast-derived TBI has also been modeled using a compressed gas shock tube to imitate a blast wave propagating through cells in the brain. This design allows for a single overpressure pulse to transmit through the sample, preventing pressure waves from reflecting off the cells (7). Compressed gas is driven down the tube and through cultured cells in inserts. It then disperses into a fluid-filled receiver below the sample chamber. Panzer et al. (7) and Effgen et al. (6) detailed the design for a blast neurotrauma device. This device has the potential to elucidate the pathology of blast-derived TBI.

While TBI is a major healthcare concern, there is currently no effective pharmacological treatment to prevent brain damage after injury. The options available, medically-induced comas or burr holes to relieve pressure, do not appear to reverse or minimize brain damage (4). Given the unmet need, an effective high-throughput method of screening potential therapeutics would thus represent an important contribution to the field.

In this study, we tested two models of TBI. In the first, TBI was modeled in vitro by adding AVP to primary neuronal cultures. We then measured the levels of the water channel protein AQP4, which is dysregulated in TBI and has been implicated mechanistically in the cellular edema that causes significant morbidity and mortality. In the second model, we mechanically simulated TBI using a blast device that sends a pressure wave through a cell
culture sample to mimic blast-induced TBI. In these studies, we started developing measurement systems that can be reliably used in future blast model investigations.

Methods

Chemical TBI induction

Astrocyte line ATCC-CRL 2541 was cultured in DMEM Gibco 10569-010 medium enriched with 8% fetal bovine serum in 6-well plates for 3 days. The experimental design consisted of three groups: 1. vehicle (dimethyl sulfoxide (DMSO)) control; 2. 10 nm vasopressin (v/v) and 3. 10 nm vasopressin and 100 nm SRX246, a selective V1aR antagonist. 2 microliters of each treatment were added to 2 mL of medium. Treatments were added to the culture when the cell population reached near the state of confluence and lasted for 5 hours. Cells were detached from the culture wells with a plastic cell scraper. The resulting suspension was centrifuged for 3 minutes at 5000 rpm at 4°C. The pellet was suspended in 200 microliters cell lysis buffer containing 20 mm Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mm MgCl2, 0.1 mm PMSF, 0.02% sodium azide and 2% SDS. The cells were lysed through sonication, which led to a clear lysate. Samples were analyzed for the contents of AQP4 through Western blot analysis. The samples were run through gel electrophoresis at 120V for 75 minutes. The gel was transferred to nitrocellulose paper and developed using Ponceau-S dye then TBS buffer with 5% dry milk for one hour. Primary and secondary antibodies were added. The nitrocellulose paper was then developed using ECL reagents and film. The film was then analyzed for band density using Scion Image. The integrated band density is the product of the band density and the band area. These values represent the signal level of the target protein, AQP4, in the samples.
Blast device and sample loading

The blast device was modeled after the design in Panzer et al. (7) and Effgen et al. (6). It consists of an aluminum driver section, an aluminum shock tube, a sample receiver, and a fluid-filled receiver. Pressurized nitrogen is released and the shockwave propagates down the tube, through the sample, and into the fluid-filled receiver to simulate a blast-derived TBI *in vitro*.

![Blast device](image)

Figure 2a. The blast neurotrauma device. The compressed nitrogen connects to the top of the shock tube, where it initiates a blast wave that bursts through plastic membranes. The pressure wave then propagates through the sample chamber, which is expanded to the right of the figure. The wave dissipates into the fluid-filled receiver after hitting the sample. The shock tube is 4 feet long and 7.5 cm in diameter. Image source: picture Amanda Sidwell; schematic Effgen et al. (6); compilation Mark DiMaggio
Mixed primary neuronal culture harvested from CF1 strain neonatal mice brains was cultured in DMEM medium enriched with 8% fetal bovine serum. After 14 days of culture in inserts in 12-well plates, the inserts were taken out of the plates and placed into bags of sterile medium. Three inserts are placed in each bag. The bags are placed in the incubator while the device is prepared for blast. To prepare the device, room temperature (37°C) water is poured into the sample receiver chamber above the fluid-filled receiver. The receiver is also filled with room temperature water. The sample bag is then placed into the sample receiver and more room temperature water is filled in to the top of the chamber. The bag is placed so that the shockwave propagates evenly through each insert. A High-Purity High-Temperature Silicone Rubber Sheet is stretched over the top of the sample receiver to eliminate air bubbles and secured. The sample
receiver is closed with metal clamps to secure it to the top of the fluid-filled receiver and the bottom of the shock tube. Circular 0.25 mm thick membranes made of polyethylene terephthalate (PET) are inserted into the top portion of the device above the shock tube. The control panel is then utilized to initiate the blast wave.

The number of membranes varies with the blast conditions: mild, moderate, and severe TBI can be imitated using different numbers of membranes. More pressure builds up with more membranes, resulting in a stronger shockwave and thus imitating a more severe injury. One membrane was the mild TBI condition, three membranes was moderate, and five membranes was severe. Pressurized nitrogen is used to burst the membranes and generate the shockwave. After the blast, the cells were retrieved and placed back into the incubator in medium until analysis. The samples in the control condition were removed from the incubator for an equal amount of time as the cells that are blasted. The cells are placed back into culture and analyzed for cell death.

Cell death analysis

Flow cytometry and beta-actin immunodetection were used to analyze cell death following blast injury with the device.
Figure 3. Schematic of overall procedure using blast device. Astrocytes cultured in inserts in well plates were brought to the blast device for blast testing. The cells were put back into culture following the experimental procedure. They were then analyzed using several methods.

Flow cytometry using propidium iodide was utilized to analyze cell death in control and blast conditions. Dead cells are marked through their higher emission wavelengths. The absorbance threshold for dead cells was $10^4$. The flow cytometer detected the amount of live and dead cells compared to the total number of events detected. The cells were analyzed the same day of the blast condition. A beta-actin Western blot was utilized for a more qualitative analysis of cell death. As a housekeeping gene present in all cells, immunodetection of beta-actin protein expression allows for the visualization of the overall number of living cells. The Western blot was used to compare the number of cells living after being blasted with one, three, or five membranes.

Results

Chemical TBI Induction

Figure 4a. Aquaporin-4 immunodetection in astrocytes. AQP4 protein expression level was assessed by Western blot in control, AVP, and AVP/antagonist conditions. The first two lanes are control conditions treated with DMSO. The middle two lanes had 10 nm AVP added. The
last two lanes are 10 nm AVP and 100 nm SRX246, a selective V1a receptor antagonist, added concurrently. AVP increased expression of AQP4 in the experimental condition, and the antagonist prevented this upregulation.

Figure 4b. Integrated band density of aquaporin-4 Western blot. Background levels of AQP4 are shown in the CTL (control) condition. 10 nm AVP upregulated the expression of AQP4 significantly from background levels. 10 nm AVP and 100 nm SRX246 showed levels of AQP4 similar to background. The V1a receptor antagonist prevented the upregulation of AQP4 following injury.

Vasopressin upregulated AQP4 (Figure 4a). Vasopressin in conjunction with a selective V1a receptor antagonist attenuated the increase of AQP4 post-injury (Figure 4a). The bottom band is 30kD, the molecular weight for AQP4. The top band is glycosylated AQP4 at 45kD, an analog of the protein. In the chemical model of TBI, the control condition measured the background levels of AQP4. 10 nm of AVP was added to the simulated injury condition. 10 nm
of AVP and 100 nm of selective V1aR antagonist SRX246 were added concurrently to the third condition. The control condition showed the background level of AQP4 in the astrocytes. In the AVP “injury” condition, AQP4 was upregulated in the presence of the hormone. SRX246 prevented the upregulation of AQP4 by vasopressin. The Western blot is quantified in the integrated band density graph (Figure 4b). The density analysis confirmed that AVP upregulated AQP4 significantly and the antagonist prevented AQP4 expression from increasing above baseline levels in the presence of AVP.

**Flow cytometry**

Figure 5a, b. Flow cytometry data for control condition in mixed primary neuronal culture. The graph (a) and table (b) show the amount of live and dead cells. $10^4$ is the absorbance threshold for a cell to be considered dead. The area under the curve to the left of the threshold represents live cells. The table (b) quantifies the amount of dead and live cells compared to the total number of cells.
Figure 5c, d. Flow cytometry data for blast condition in mixed primary neuronal culture. The graph (a) shows qualitatively an increased number of dead cells above the fluorescence threshold. The table (b) indicates a higher dead to live cell ratio, but fewer overall events detected.

Figure 5e. Graphical representation of cell death data for control and blast conditions at the time of flow cytometry analysis. Live and dead cells are shown as a percentage of the total parent cell population of their respective sample condition.

The control condition resulted in an overall greater count of both live and dead cells (Figure 5a, b). The blast condition showed fewer total cells counted, but a proportionally larger number of dead cells (Figure 5c, d). For the control condition, 653 events were counted as debris,
or unreadable by the machine. Of the 9,347 cells detected, 8,121, or 86.9% of them were live. 13.1% of the cells detected met the fluorescence threshold to be considered dead. 10,000 total events were counted. For the blast condition, 63.8% of the 4,247 cells were live while 36.2% were dead. While this represents a higher ratio of dead to live cells, this number should be viewed cautiously as only 5,525 total events were counted in the flow cytometry protocol.

**Beta-actin Western blot**

![Beta-actin Western blot](image)

Figure 6. Immunodetection of beta-actin in astrocytes. The first two lanes are a control condition where the cells were not blasted. The second two lanes were blasted through one membrane. The fifth and sixth lanes had 3 membranes. The final two lanes were blasted through five membranes.

Beta-actin is a housekeeping gene present in all cells. Immunodetection of beta-actin was used to evaluate cell death in control, one membrane, three membrane, and five membrane conditions. Beta-actin was detected at 43kD, its molecular weight. Greater diaphragm thickness contributes to higher blast pressure. The five-membrane condition showed a thinner band, indicating fewer cells. This suggests that the device is effective at the five membrane and above condition. These results will be used to further refine the procedure and experiment with higher numbers of membranes in the device.

**Discussion**

**Conclusions**
The chemical TBI induction experiments confirmed injury-related upregulation of AQP4 by AVP. SRX246, the selective V1a receptor antagonist, was able to attenuate increased AQP4 expression in the presence of AVP. However, this procedure did not show promise as a valid, replicable model. The Western blot results that demonstrated the expected outcomes could not be replicated. Numerous variables were modified to attempt to repeat the results. The sonication time was altered to create a more uniform pellet for the Western blot analysis. Fresh reagents were used for each step of the process. Cells were grown to various states of confluence to create stronger signals of protein expression. Despite these modifications to the procedure, the promising results produced could not be repeated. The lack of replicability of these results led us to create the blast device to more accurately imitate the conditions of TBI in the brain.

The blast device was designed to simulate blast TBI in vitro. It was designed after the blast neurotrauma model outlined in Panzer et al. (7) and Effgen et al. (6). Cells were blasted using pressurized nitrogen and analyzed for cell death using a beta-actin Western blot and flow cytometry. Beta-actin immunodetection showed that the device injures cells. The thinner band in the five-membrane condition indicates that fewer cells survived in the blast condition than in the control condition. Flow cytometry data indicates the blast device results in significantly more dead cells in the blast condition than in the control condition using five membranes. Future studies involving higher numbers of membranes are necessary to precisely quantify cell death after blasts. The blast neurotrauma model has potential as a high-throughput screening mechanism for drugs to combat brain damage from TBI. The V1a receptor antagonist diminishes the expression of AQP4 following injury. This is promising to prevent cellular edema, as shown in Figure 1. Interrupting the secondary edema signaling cascade post-TBI would prevent widespread cellular swelling and potentially mediate damage.
Refinement of procedure

The flow cytometry data indicated that fewer cells survived the blast condition than the control condition. Proportionally, 36.2% of cells died in the blast condition compared to 13.1% in the control condition. Astrocytes in culture spread out, appearing to grip the insert in which they are cultured. When these cells die, they round up and no longer adhere to the insert. During the re-culture step post-blast, the dead astrocytes may be lost in the medium and thus become undetectable by the flow cytometer. This is a possible explanation for the 5,525 events detected in the blast condition compared to the 10,000 of the control condition. Loss of dead cells before analysis would skew the number and ratio of live to dead cells in the blast condition compared to the control.

The three inserts in each bag during the blast condition may alter the precise pressure received by each sample. A different angle in relation to the blast wave could cause a different amount of pressure to reach each portion of the insert, or each insert as a whole. Ensuring that each insert is directly upright while in the bag in the sample chamber poses difficulty. The angle and position of each insert may alter the exact shockwave each insert receives, potentially leading to variation in cell death numbers. The temperature of the water in the device could stress the cells and cause some additional cell death in the blast condition. Using warmer water is difficult because of the size of the receiver and maintaining constant temperature in the sample receiver. By altering these variables, we may get more accurate results from the blast neurotrauma device.

Future directions
The installation of a pressure quantifier at the top of the device will allow for precise pressure measurements. Correlating exact kPa of pressure with each blast membrane condition will allow for a more accurate depiction of how hard the cells are struck to cause death or damage. The pressure transducers will be installed at the top of the shock tube and on either side of the sample in the receiver of the device to measure the intensity of the shockwave. They can record the burst pressure when the membranes break from the shockwave. The speed of the shockwave can be measured by the time elapsed between the wave bursting the membranes and reaching the pressure transducers below the sample. This dose-response relationship of pressure to injury will allow for more precise experiments.

The use of brain slices will more accurately simulate the effects of TBI on the brain as a whole. This simplified blast injury model allows for the study of biological outcomes in vitro (6). Utilization of biomarkers specifically representative of physiological changes related to cell death will aid analysis of blast conditions. Analyzing samples for cell death at different time points will further elucidate the time course of vasopressin secretion and AQP4 upregulation after injury. After the parametric studies of the device are complete, it has the potential to be used as a screening tool for TBI therapeutics. Future studies will examine the efficacy of V1a receptor antagonists in reducing the expression of AQP4 post-injury. Time-course and dose-response studies involving these drugs are necessary.

References


