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Development of a semi-interpenetrating network hydrogel to study the effects of mild traumatic brain injury on astrocyte remodeling

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of the requirements for the degree of
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by

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Abstract

Traumatic brain injuries (TBIs) are a widespread public health concern affecting over 2.5 million people in the United States alone (1). TBIs are the leading cause of death and disability in children (ages 0-14), and their devastating effects can also be seen in vast population subsets such as professional athletes and combat soldiers (1).

Damaged astrocytes, the specialized glial cells associated with the maintenance of the brain's environment, respond to injury by altering regulation of certain proteins and ion channels in an attempt to maintain homeostasis (3). Studies have also shown that following a TBI, astrocytes seek to regenerate, a phenomenon known as astrocyte reactivity. The purpose of this study was to develop a semi-interpenetrating network hydrogel to examine the expression astrocyte injury marker S100 calcium-binding protein B (S100B) and astrocyte reactivity marker glial fibrillary acidic protein (GFAP) following a mild TBI.

To begin, I used a bioreactor designed by a previous student to simulate a compressive TBI. Following this, I analyzed the expression of S100B and GFAP on the astrocyte cells using real-time PCR. I also examined the effect of laminin on the astrocyte injury. Multiple issues were encountered throughout the course of the project. Challenges with cell culture and contamination were actively troubleshooted throughout the semester; however, despite the extensive investigations which are detailed in this paper, no clear solution was developed.

The results of this project were inconclusive due to small sample size. Because S100B and GFAP are secreted following injury, our experiments suggest that the expression of these genes in the astrocytes were lower post-TBI. It also appears that laminin assists in protecting the astrocytes from extensive injury. In order to gain more accurate and conclusive results, the experiment sample size must be increased.

Understanding the roles of these markers in relation to astrocyte injury is vital to the development of more effective tertiary treatments of TBIs and positive clinical outcomes.

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Chapter 1

1. Introduction

Traumatic brain injuries (TBI) are a widespread public health issue affecting many populations in the United States, ravaging the lives of 2.5 million people annually (1). TBIs potentiate intracranial damage due to a large force to the head such as a blow or jerk. The immediate effects of a TBI include bleeding, swelling, memory loss, and headache (2). TBIs are classified on a scale based on severity. Mild TBIs are the most common and occur when one remains unconscious or disoriented for less than 30 minutes (12). Common symptoms of mild TBIs (mTBIs) are fatigue, headache, irritability, memory loss, and sleep disturbances (12). Severe TBIs, in which loss of consciousness lasts longer than six hours, may also cause damage to brain tissue and long-term neurological disabilities resulting in limited functioning of the body (2).

Immediately following a TBI, the brain and its surrounding tissues undergo a series of major alterations, contributing to cell apoptosis and further neuron damage (3). There are two stages to the pathophysiology of a TBI. The primary damage from a TBI occurs directly at the moment of impact and includes such injuries as lacerations, bruising, and bleeding (6). Perhaps the more worrisome and unpredictable damage occurs secondary. This indirect damage occurs post-impact and includes edema, ischemia, and hypotension (6). Mild TBIs can cause memory loss, headache, sleep disturbances, and irritability (12). Moderate and severe TBIs can lead to hemorrhaging of the brain, memory loss, cell apoptosis, and even permanent paralysis.

Following a TBI, the surrounding tissues of the brain undergo a series of major alterations in an attempt to maintain homeostasis within the central nervous system (4). Astrocytes and other cells that are involved in the maintenance of homeostasis within the brain's environment alter gene expression after a TBI. This alteration in the internal environment of

astrocytes contributes to the cell death and larger, more systemic damage of the brain and spinal cord. Two important genes that are involved in this process are S100 calcium-binding protein B (S100B) and glial fibrillary acidic protein (GFAP). Previous research has shown that S100B is expressed in response to cell damage in astrocytes; thus, it is traditionally used as a marker for cell injury (7). Furthermore, S100B has been shown to be upregulated following astrocyte injury (7). Additionally, studies have also shown that following a TBI, astrocytes seek to regenerate, a phenomenon known as astrocyte reactivity. GFAP is used to characterize astrocyte reactivity after cell injury (8). GFAP is involved in the proliferation of astrocyte cells post-TBI, contributing to the formation of glial scars to facilitate the astrocytic healing process (8).

1.1 Objectives:

My main focus was to examine the expression of astrocytic GFAP and S100B following a simulated TBI. The hypothesis of this study was that TBIs will lead to an increased expression of both S100B and GFAP in astrocyte cells. Additionally, it was also hypothesized that the inclusion of laminin, a brain specific extracellular matrix protein, would confer possible protective effects on astrocyte cells. To address this hypothesis, I subjected the astrocytes to a high-speed compression that mimics the strain experienced by the cells during a TBI by utilizing an in-house bioreactor designed by a previous research team member. Following the TBI, gene expression was measured using quantitative real-time PCR.

1.2. Significance of Research

This research pertaining to traumatic brain injury will advance the understanding of S100B and GFAP expression after astrocyte injury. By further characterizing the specific transformations that occur following a TBI, more effective treatments of TBIs and related

neurological injuries can be explored. Furthermore, the experiences and techniques that I have gained from this research project have increased my critical thinking skills, problem-solving ability, and application of knowledge.

1.3 Abbreviations and Definitions:

BBB – blood-brain barrier
- Semi-permeable membrane surrounding brain
- Provides protection from substances and controls what enters the brain

TBI – traumatic brain injury
- Intracranial damage caused by jerk or blow to the head

mTBI – mild traumatic brain injury
- Unconsciousness or disorientation lasting less than 30 minutes
- Most common type of TBI

GFAP – glial fibrillary acidic protein
- Provides support and strength for cells
- Marker for astrocyte reactivity

S100B – S100 calcium-binding protein B
- Evaluates astrocytic damage

Astrocyte or astrocyte cells
- BBB-supporting cells
- Likely to be damaged after TBI

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Chapter 2

2. Literature Review

2.1 Traumatic Brain Injury:

TBIs are a major cause of death and disability in the United States (1). Annually, of the 2.5 million reported TBIs, 50,000 people died and 280,000 people were hospitalized (1). These numbers are most likely an underestimate, as they do not account for persons treated for TBIs both in US military hospitals and abroad. The CDC estimates that in 2010 alone, the economic costs associated with TBIs were $76.5 billion (1). With this vast economic, social, and health burden, it is notable that most public health programs currently focus on the prevention of TBIs as the primary mechanism to control the issue.

The leading cause of TBIs are falls, accounting for almost half of reported TBIs (1). The rates of TBIs are highest for persons aged 65 or older, and men are three times more at risk than women of suffering a TBI (1).

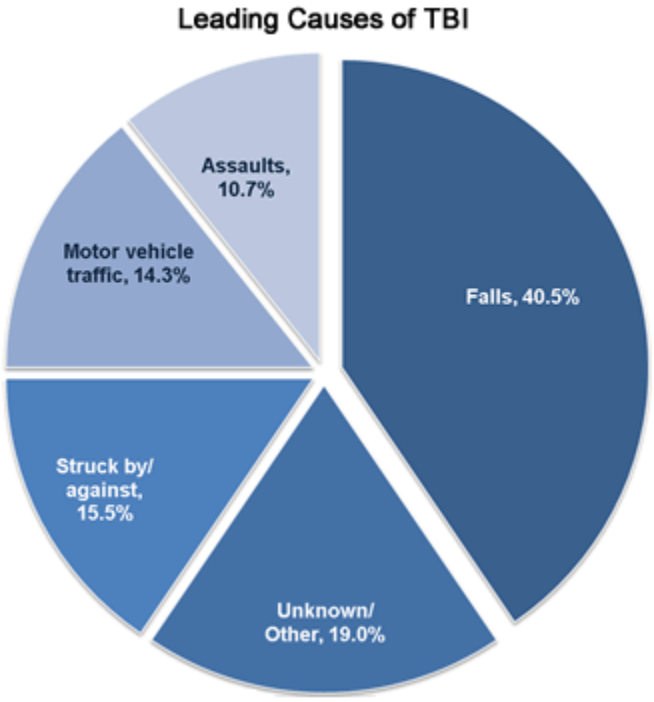

Figure 1: Breakdown of leading causes of TBIs (1).

Currently, the clinical treatments of TBIs lag behind the vast impact of the injury. Once a TBI has occurred, little can be done to reverse the damage, and existing treatments focus on minimizing secondary damage (5). Patients may be given diuretics to control blood pressure, anti-convulsants to prevent seizures, and coma-inducting medications once they received medical care (5). After damage assessment, patients may also participate in rehabilitation including speech, occupational, physical, and cognitive therapy programs (5).

Though the public health campaigns focusing on primary prevention have been effective, there is now a vital need for tertiary prevention to reduce the burden of TBIs and improve clinical outcomes. Thus, it is currently important to focus research efforts on understanding the mechanisms behind the changes that surrounding brain tissues undergo directly following a TBI in order to develop new clinical treatment regimens.

2.2 GFAP and Reactive Astrogliosis:

Glial fibrillary acidic protein (GFAP) is a protein which provides support and strength to cells (9). Although not fully understood, GFAP is most likely involved in supporting astroglial cells through maintaining shape, support, and function (9). Following such injuries as ischemia, trauma, and neurological conditions, astrocyte cells will respond with the development of new astrocytic cells, known as reactive astrogliosis (8). Reactive astrocytes demonstrate altered gene expression and distinct morphological features. In previous studies, GFAP has been shown to be upregulated during reactive astrogliosis and is considered a marker activated astrocytes (10).

Preliminary laboratory experiments have shown an increase in the proliferation of astrocytes 7 days post-TBI. In order to further characterize this proliferation, we used GFAP as a marker of reactive astrogliosis.

2.3 S100B:

S100 calcium-binding protein B (S100B) is involved in the assessment of cell injury (8). It is expressed by mature astrocytes and is able to be isolated from serum in order to appraise the severity of brain damage (11). Following a TBI, S100B gene expression should increase because of the trauma associated with the impact.

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Chapter 3

3. Materials and Methods

3.1 Cell Culturing:

Primary astrocytes isolated from the brain of neonatal rats were cultured in Dulbecco's Modified Eagle Medium (DMEM) 10% fetal bovine serum (FBS) (including pen/strep and hepes) in T-75 flasks at 37°C and 5% CO_2. The cells were utilized once they reach 85% confluence in preliminary culture.

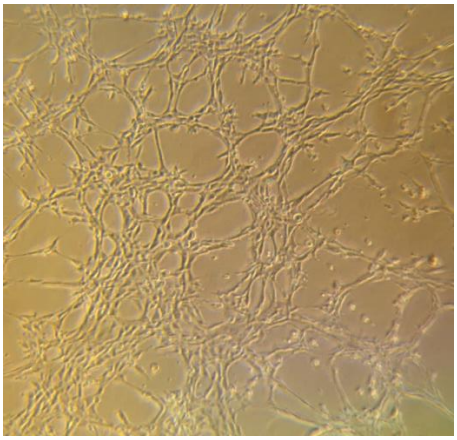

Figure 2: Example of astrocytes during cell culture.

For the TBI experiments, the cells were first suspended in a mixture of alginate, FBS, and 3X DMEM. These cultured cells were then seeded onto a calcium chloride agar stamp to solidify overnight; the calcium chloride in the agar stamp allowed the gel to maintain a crystalline structure. This creates a 3D model that could withstand the simulation of a TBI. Laminin was also added to half of the cells to evaluate the effect of laminin on astrocyte injury. Figure 3 shows the 12-well plate set-up for each sample.

Day 1:

Control		**TBI**	
(-) Laminin	(+) Laminin	(-) Laminin	(+) Laminin

Day 7:

Control		**TBI**	
(-) Laminin	(+) Laminin	(-) Laminin	(+) Laminin

Figure 3: Demonstrates the 12-well plate arrangement.

Throughout the course of this project, our astrocyte cells became contaminated multiple times during cell culture. In order to identify the source of the contamination, we performed the following tests (Figure 4):

Suspected Source	**Actions**	**Outcome**
Media	• Refiltered media • Utilized different medias on half of the cells	Unsuccessful
PBS	• Refiltered PBS	Unsuccessful
Breakdown in aseptic technique	• Replaced person responsible for cell culture	Unsuccessful
Incubator	• Removed cells, sterilized incubator	Unsuccessful

Figure 4: Discusses actions taken to identify source of contamination during cell contamination

3.2 TBI Simulation:

24-hours after seeding the cells in suspension, I separated the gels from the agar stamp and added culture media. One day later, I compressed the cells using a bioreactor (see Figure 5), simulating the force that the brain undergoes when a TBI occurs. One day after compression, I isolated the RNA using the Qiagen RNeasy Isolation Kit, sequestering this for future gene analysis. I utilized this same procedure on another set of cells seven days post-compression.

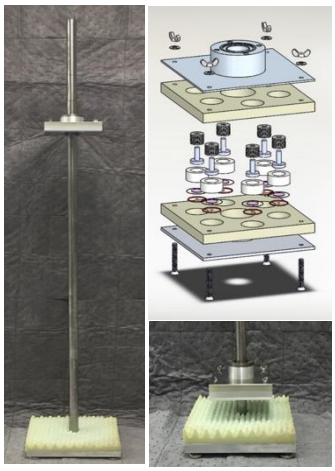

Figure 5: Bioreactor device that was used to simulate a TBI.

We also experienced contamination of the gels. In order to identify the source of the contamination, the following techniques were used (Figure 6):

Suspected Source	**Actions**	**Outcome**
Alginate	• Sterilized alginate before use	Successful
Laminin	• Refiltered laminin before use	Unsuccessful

Figure 6: Discusses actions taken to identify source of contamination during gel construction

3.3 Gene Expression:

After isolation of the mRNA, we utilized reverse transcriptase to convert the samples into cDNA. This is more stable than mRNA, ensuring the integrity of the sample is maintained during storage. Following this, real-time PCR was completed on the sample using the primers S100B and GFAP. Each well was loaded with sterile water, SYBR Green, appropriate primer, and respective sample. GAPDH was used as our control. GAPDH is ubiquitous and is very stable throughout environmental strains such as compression.

3.4 Pictorial Diagram of Methods:

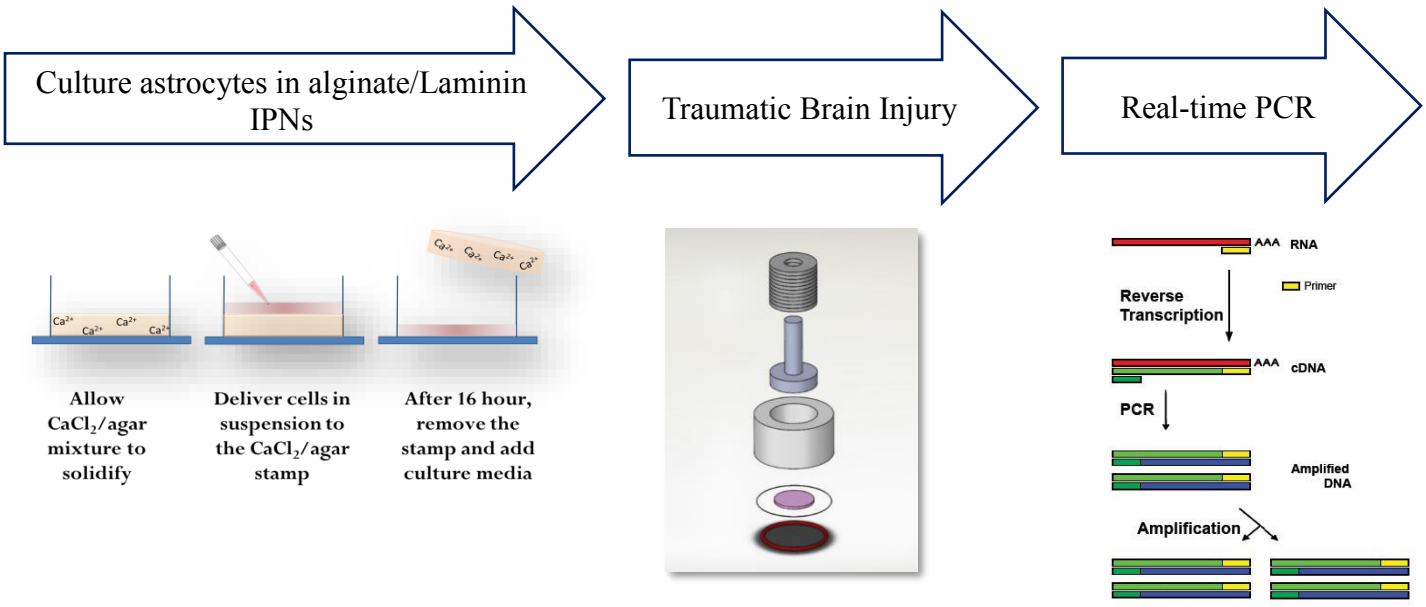

Figure 7: Provides a brief pictorial diagram of the methods.

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Chapter 4

4. Macro Perspective

My examination of S100B and GFAP was a smaller aspect of Nasya Sturdivant's, a PhD candidate of Dr. Kartik Balachandran, dissertation. The overall goal of her dissertation is to study how traumatic brain injury affects the brain and blood-brain barrier (BBB). To develop new therapeutic interventions, it is necessary to further characterize the changes that occur post-TBI. As such, Nasya is investigating multiple proteins that may cause the secondary injury following a TBI. I examined the specific genes S100B and GFAP which Nasya would like to include in her dissertation.

Additionally, another main goal of Nasya's thesis is to develop a new model for TBI simulation. During a TBI, multiple events occur including stretch, compression, impact, collision, and jolt. Because of the complexity of this event, simulating it accurately and holistically in the laboratory presents challenges. To establish TBI simulations which resemble more realistic events, Nasya is also examining multiple models for TBI simulation by studying the impact of the type of force (ie stretch or compression) and environment (ie 3-D model or 2-D model). The bioreactor compressed the astrocytes, paralleling the 3-D model with a compression force.

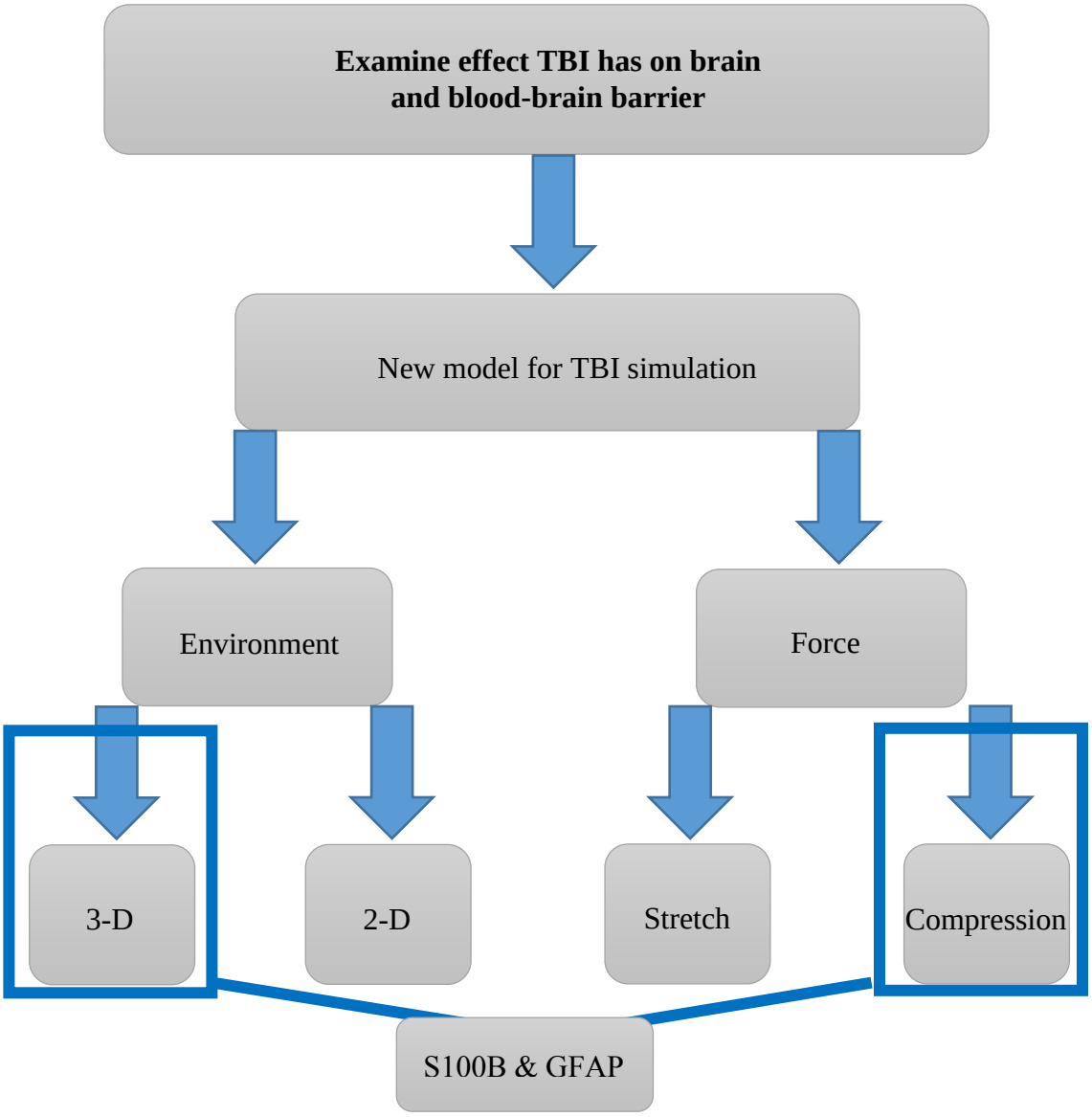

Figure 8: Provides a visual diagram of the macro-perspective on my honors thesis project.

5. Results and Discussion

Through real-time PCR, the genes S100B and GFAP were analyzed. S100B is a gene used to examine cell injury (8). Following a TBI, we expect astrocyte cells to exhibit more injury and thus more S100B than in the control. Secondly, GFAP is a gene that is a marker for reactive astrogliosis (8). After a TBI, astrocyte cells grow and proliferate rapidly to form a glial

scar (10). Because GFAP has been correlated with the formation of a glial scar, we expect GFAP to also increase following a TBI.

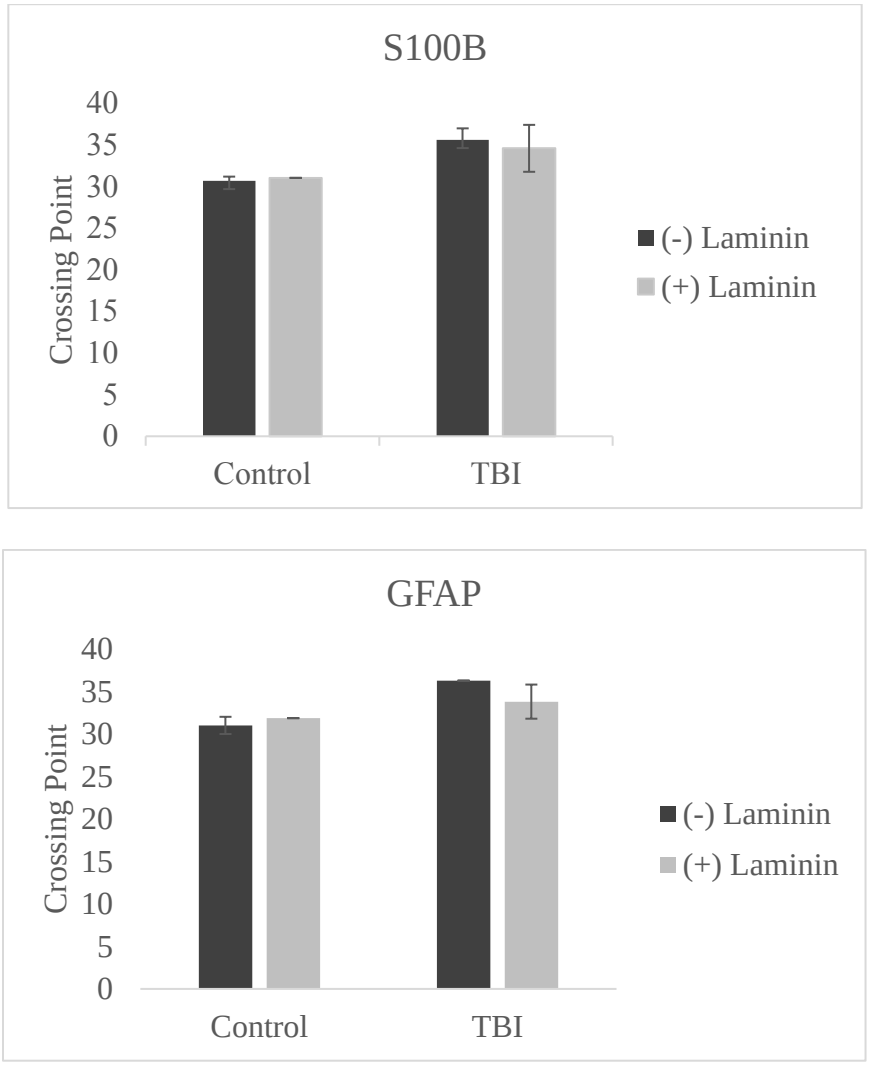

Figure 9: Demonstrates the results of real-time PCR analysis for S100B and GFAP

Figure 9 displays the results of the real-time PCR analysis. The y-axis displays the crossing point value at which the gene reaches a threshold during amplification. The x-axis represents the control and TBI samples with and without laminin. Following a TBI, S100B appears to increase the crossing point value, meaning that the amount of S100B in the cell decreased. Larger crossing point values suggest a longer time to reach the threshold value,

meaning that more amplifications cycles are required and thus less of the gene is present. GFAP also has a larger crossing point value after a TBI, indicating that it too decreased in the astrocyte cells following a TBI. The sample sizes and standard deviations for each are below:

S100B

Sample	**Sample Size**	**Standard Deviation**
Control (+) Laminin	1	n/a
Control (—) Laminin	2	0.70711
TBI (+) Laminin	2	3.98808
TBI (—) Laminin	2	1.94454

GFAP

Sample	**Sample Size**	**Standard Deviation**
Control (+) Laminin	1	n/a
Control (—) Laminin	2	1.43189
TBI (+) Laminin	3	3.48031
TBI (—) Laminin	1	n/a

Figure 10: Describes sample size for each gene

GFAP and S100B are both genes that are also used as serum biomarkers following injury (8). Because these are detectable in the bloodstream, astrocyte cells secrete them post-TBI. This secretion following injury explains the lower values in the astrocyte TBI samples. The astrocyte cells released these biomarkers into the extracellular environment after the injury, causing the lower detection inside the cells. The laminin seems to have provided protective factors for the astrocyte cells as the samples with laminin expressed less injury and reactive astrogliosis. However, because of the low sample size, conclusions cannot be drawn based on this data.

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Chapter 5

6. Individual Skills Attainment

6.1 Laboratory Skills:

Throughout this project, I gained a multitude of laboratory skills. I have listed below the laboratory bench skills which I developed through my involvement in my honors thesis project and in the laboratory:

- Cell culture
- Primary cell isolation
- RNA isolation
- Real-time PCR
- Project development

6.2 Professional Development Skills

In addition to laboratory bench skills, I gained experiences and developed skills which I will carry into the workplace. Most importantly, my research provided me with a physiological perspective on disease. This micro-biological perspective will enhance my future career by allowing me to grasp physiological disease progression and specific laboratory diagnostic methods. Additionally, I learned through failed trials and project challenges that research requires methodical and persistent dedication to a task. I will carry this tenacity into my future in medical school and as a physician. Furthermore, because clinical treatments originate from various versions of the scientific method, I will be better prepared as a physician to be a humble lifelong learner who routinely and systematically seeks improvements in my work and in my methodology.

6.3 Soft Skills

During the semester, I also gained the soft skills listed below:

- Problem solving

- Critical thinking
- Information literacy through academic journal articles
- Collaborative skills
- Written communication skills
- Oral communication skills
- Data analysis
- Creativity
- Organization

7. Conclusion and Future Work

Because the sample size was low, no significant conclusions can be drawn from the results. The sample size must be increased in order to see significant differences between the control and TBI astrocytes.

In the future, we would like to increase the sample size to five for each respective sample. Additionally, three other markers for reactive astrogliosis will be examined in future studies including vimentin, IL-6, and TNF-alpha. In order to sequester purer mRNA from each sample, it is important in future trials to maintain a sterile environment by wearing masks and lab coats to prevent any outside RNA contamination. Additionally, strict use of RNAse Away is vital to maintain the integrity of the mRNA samples during handling. Lastly, mRNA should be converted to cDNA directly after it is isolated and before it is frozen; this allows the sample to be more stable during storage.

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Appendix

A. Cell Culture

Seeding Cells from Frozen Vials

Reagents and Materials:

Reagent	Notes
DMEM-10% FBS	From pg. 1 of protocol (or other appropriate media type)
Frozen vial of cells	Retrieve from liquid N_2 dewar
Dulbecco's phosphate buffered saline (dPBS)	Invitrogen #14190250
Cell culture plate	Use T-25, T-75, T-175, P-60 etc as appropriate General rule of thumb: 50,000 cells per cm^2 will yield confluent culture

Coating cell culture plates:	
1% gelatin (if needed)	Dissolve 1g gelatin (Sigma #G2500) in 100mL boiling water. Cool and sterile filter.
1-50µg/mL fibronectin	Dissolve 1mg fibronectin (BD Biosciences # 354008) in 1mL **cold** ***sterile*** water (will yield: [FN] = 1mg/mL). Prepare 100µL aliquots and store in -80°C freezer.

Protocol:

1. Prepare your substrates:
    a. Spray and transfer cell culture plate(s) into biosafety hood.
    b. Coat plates if your protocol calls for it.
2. Warm up all reagents in the water bath (37°C). ***Warm frozen vial only to the point when the last sliver of frozen cells has melted.***
3. Spray down with EtOH and transfer to Biosafety Hood.
4. Transfer contents of cell vial to 15mL/50mL conical tube and top up with at least 10 times the volume of media (eg: 10mL DMEM-10% FBS for 1mL frozen cells).
5. Spin down in centrifuge at 4°C. A good starting point is 200xg for 5 min.
6. In the meantime, aspirate cell culture coating and wash 3x with dPBS.
7. Once cells have been centrifuged, return to biosafety hood and carefully aspirate media above the cell pellet. ***Take care that you don't accidentally aspirate the cells!***
8. Gently resuspend the cells in appropriate volume of DMEM-10% FBS. Use about 5-10 passes of the pipet to mix the cells evenly in the media.
    a. T-25: 2mL
    b. T-75: 10mL
    c. T-175: 25mL
9. Remove 10µL of this solution for counting the number of cells.

10. Pipet cell solution to cell culture plate and gently tilt plate to ensure complete surface coverage of the cell solution.
11. Label plate with: Your name/initials, date, cell type, passage number (will be found on frozen vial).
12. Place cell culture plate in incubator at 37°C, 5% CO_2.
13. Allow cells at least 24 hours to attach to plate before feeding them. Feed cells every 2-3 days.

Changing Media/Feeding Cells

Reagents and Materials:

Reagent	Notes
DMEM-10% FBS	From pg. 1 of protocol (or other appropriate media type)
Dulbecco's phosphate buffered saline (dPBS)	Invitrogen #14190250

Protocol:

1. Spray down microscope stage and examine cells. If cells look about 75-90% confluent, they need to be passaged.
2. Warm up all reagents in the water bath (37°C).
3. Spray down with EtOH and transfer to Biosafety Hood.
4. Gently aspirate old cell culture media. Make sure you aspirate media from the edge of the flask. Tilt flask to facilitate media aspiration.
5. Working quickly to prevent drying out of cells, wash cells 3x using dPBS.
6. Gently tap flask against work surface of biosafety hood (if needed).
7. After final wash, aspirate dPBS and add appropriate amount of DMEM-10% FBS.
    a. T-25: 2mL
    b. T-75: 10mL
    c. T-175: 25mL
8. Place cell culture plate in incubator at 37°C, 5% CO_2.
9. Continue monitoring cells under microscope every day.

Sub-culture/Passaging of Cells

Reagents and Materials:

Reagent	Notes
DMEM-10% FBS	From pg. 1 of protocol (or other appropriate media type)
Dulbecco's phosphate buffered saline (dPBS)	Invitrogen #14190250 ***(must not contain Ca^{2+} or Mg^{2+})***
Trypsin-EDTA	Invitrogen #25300054 (0.05%) Invitrogen #25200056 (0.25%)
Cell culture plate	Use T-25, T-75, T-175, P-60 etc as appropriate General rule of thumb: 50,000 cells per cm^2 will yield confluent culture

Protocol:

1. Determine the ratio of your passage/sub-culture. Typically one splits cells 1:2 or 1:3.
2. Prepare required number of substrates as per pg. 2:
3. Warm up all reagents in the water bath (37°C).
4. Spray down with EtOH and transfer to Biosafety Hood.
5. Gently aspirate old cell culture media. Make sure you aspirate media from the edge of the flask. Tilt flask to facilitate media aspiration.
6. Working quickly to prevent drying out of cells, wash cells 3x using dPBS. **This step is extremely important!!!**
7. Add appropriate volume of Trypsin-EDTA to disrupt cell-substrate and cell-cell binding.
    a. T-25: 0.5mL
    b. T-75: 1mL
    c. T-175: 2.5mL
8. Return flask to incubator for 2min. After 2 min, check cells under microscope, they should have started to ball up and detach from the plate. Tap flask if necessary to dislodge the rest of the cells.
9. Quench trypsin-EDTA reaction with 10-times the volume of DMEM-10% FBS.
10. Using a pipet transfer contents to a 15mL/50mL conical tube.
11. Spin down in centrifuge at 4°C. A good starting point is 200xg for 5 min.
12. In the meantime, aspirate cell culture coating and wash 3x with dPBS.
13. Once cells have been centrifuged, return to biosafety hood and carefully aspirate media above the cell pellet. ***Take care that you don't accidentally aspirate the cells!***
14. Gently resuspend the cells in appropriate volume of DMEM-10% FBS. Use about 5-10 passes of the pipet to mix the cells evenly in the media.
15. Remove 10µL of this solution for counting the number of cells.
16. Divide cell solution evenly to desired number of cell culture plates and gently tilt plate to ensure complete surface coverage of the cell solution. Top up with additional DMEM-10% FBS if needed.
17. Label plates with: Your name/initials, date, cell type, passage number (make sure you increment the passage by 1).

18. Place cell culture plates in incubator at 37°C, 5% CO_2.
19. Allow cells at least 24 hours to attach to plate before feeding them.

Culture Media Preparation

Reagents and Materials:

Reagent	Notes
DMEM	Invitrogen #11995073 (contains L-glutamine)
Fetal Bovine Serum (FBS)	Invitrogen #10082-147 (50mL aliquots; -20°C freezer)
Pen-strep or antibiotic-antimicotic (100x)	Invitrogen #15240-062 (5mL aliquots; -20°C freezer)
HEPES (1M)	Invitrogen #15630080
Other growth factors if required	***This is typically based on your own modified protocol, experience, or the literature***
500mL vacuum filtration unit	

Protocol:

1. Warm up all reagents in the water bath (37°C).
2. Spray down with EtOH and transfer to Biosafety Hood.
3. Remove required amount of DMEM and dispose (eg: for DMEM-10% FBS remove 60mL).
4. For DMEM-10% FBS add 50mL FBS, 5mL pen-strep, 5mL HEPES (i.e. bring the total volume back to 500mL)
5. Swirl bottle to ensure proper mixing, and filter liquid using vacuum filtration unit.
6. Immediately aliquot sterile, filtered DMEM-10% FBS into ten 50mL conical tubes.
7. Label, initial, date tubes, and return to 4°C refrigerator.
8. Use all aliquots within 2 weeks.
9. After 2 weeks, dispose of any unused aliquots and prepare fresh media.

B. 3D Cultures in Alginate

Part 1

Making 3D Alginate Gels for Mechanical Testing (Modulus, Swelling Characteristics, etc.) and SEM imaging. This protocol outlines the procedure to prepare 3D alginate gels at an alginate concentration of 0.1%, 0.5%, 1%, and 5% using 60mM and 100 mM $CaCl_2$/agar stamps. The $CaCl_2$ in the $CaCl_2$/agar stamps act as a crosslinking agent, causing the alginate solution to stiffen.

Reagents	Notes
Alginic Acid Sodium Salt from brown algae (Alginate)	Dissolve alginate in ddH20 to achieve the desired concentration. Vortex and set in water bath to aid dissolution.
On Chemical Shelf	0.1%: 0.1g alginate in 100 mL ddH_20
	0.5%: 0.5g alginate in 100 mL ddH_20
	1.0%: 1g alginate in 100 mL ddH_20
	5.0%: 5g alginate in 100 mL ddH_20
5% Agar Solution	5g agar in 100 mL ddH_20
1 M $CaCl_2$ Solution	There is usually some already made in the 4°C or on the chemical shelf, if not, the powder can be located on the chemical shelf. Dissolve in ddH20.

$CaCl_2$/agar stamps

The amounts below make one 6 well plate of stamps (50 mL/plate)

60 mM $CaCl_2$/agar stamp solution	3 mL $CaCl_2$ + 47 mL 5% agar solution
100 mM $CaCl_2$/agar stamp solution	5 mL $CaCl_2$ + 45 mL 5% agar solution

Make the stamp solution in a beaker, add a stir bar and loosely cover with foil. Boil the stamp solution on a hot plate at 90°C with the stir bar rotating. Where a protective glove and remove the solution from the hot plate and immediately begin pouring it into the wells of the 6-well plate. Fill each well up to about ¼ - 1/3 full. Let the plates cool to room temperature and then place in the 4C until you are ready to add the alginate.

Pour 2-3 mL of 0.1%, 0.5%, 1% or 5% alginate solution on the $CaCl_2$ stamps. Leave in the 37°C oven on the middle black table overnight. The alginate gels can now be removed from the $CaCl_2$/agar stamps and used for viscoelastic property measurements using the rheometer.

Prep for Swelling Tests and SEM
Remove the alginate gels from the CaCl2/agar stamps and place in a petri dish. KEEP TRACK OF WHICH GEL IS WHICH. Prep the lyophilizer. Completely freeze samples with liquid N_2.

Store samples in the -80°C until the lyophilizer is ready. Place samples in 50 mL conical tubes caped with a KEM wipe with tape wrapped around it. Leave in lyophilizer overnight.

Swelling Tests: Cut the pieces so they have similar masses. Record each pieces dry weight. Add 3 mL to each well of a 12 well plate. Put one piece in each well of a 12 well plate. Record the mass of each piece for 3 hours over a 12 hour period.

SEM Imaging: Mount samples on studs using carbon tape and follow the steps listed below.

Part 2

From the mechanical testing we discovered that when 0.5% alginate is crosslinked with 60 mM $CaCl_2$/agar stamps it creates a hydrogel with a storage modulus close to that of the brain. For this reason, the cell viability staining, osmolality tests, and protein expression will be done on astrocytes encapsulated in 0.5% alginate crosslinked with 60 mM $CaCl_2$/agar stamps.

Alginate Prep

1. Prepare 1.765% (for 1%) alginate in sterile deionized water.
2. Adjust pH of alginate to 7.4 using NaOH (basic) and HCl (acidic).
3. Filter the alginate in the cell culture hood. Place in 4°C until ready for use.

3D Cultures of Cells in Alginate Gels

1. Calculate the total volume of alginate solution needed:
   3mL/chamber

Reagent	Notes
DMEM (3X concentrated)	Sigma #D7777 (Reconstitute the powder (Entire "1L" bottle) in 333mL ddH_2O + 3.7g Sodium Bicarbonate; Filter sterilize; Aliquot to 15mL tubes and freeze in -20°C) $\text{Volume} = \text{Total Volume} \div 3$ DMEM Volume = Total Volume ÷ 3 (Use the 5mL filtered aliquots in -20°C)
Fetal Bovine Serum (FBS)	Invitrogen #10082-147 (50mL aliquots; -20°C freezer) FBS Volume = Total Volume × 0.1 (To make the solution 10% FBS)
0.8825% Sterile Alginate	Alginate Volume = T.Volume – DMEM – FBS

2. Trypsinize cells. Count. Remove needed amount of cells (500,000 cells/chamber) and spin down. Re-plate the remainder cells.

3. Resuspend cells in the appropriate amount of 3X DMEM. Then, add in FBS and Alginate.
4. Mix the solution well but take care to avoid the generation of bubbles.
5. Immediately transfer 3mL of the cell suspension into each well of the 6-well plate with the $CaCl_2$/agar stamps.
6. Allow the solution to gel overnight at 37°C, 5% CO_2, 100% humidity (i.e. in the incubator)
7. Feed the gel with 2 mL normal DMEM-10% FBS

Note: To make a gel with an alginate concentration other than 1%, multiply the desired alginate concentration by 1.765. Example: For a 5% alginate gel, multiply 1.765 by 5. You will now create your alginate solution with a 8.825% alginate, yielding a gel with a final alginate concentration of 5%.

C. Purification of Total RNA from Rat Astrocyte Cells

Checklist

- Spray countertop, pipetters, pipet boxes, 2 microcentrifuge tube holders, etc. down with DNase/RNase Away.
- **Grab the Buffer RW1.**
- **Prep Buffer RLT Plus**. Make sure β-mercaptoethanol (β-ME) was added to Buffer RLT (check on the bottle top). If not, Add 10 uL β-ME per 1 mL Buffer RLT Plus. Buffer RLT Plus is stable at room temperature for 1 month after the addition of β-ME
    - Buffer RLT Plus may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- **Prep Buffer RPE working solution**. Make sure ethanol has been added to Buffer RPE to create the working solution (check on the bottle top). If not, add 4 volumes of ethanol (96-100%), as indicated on the bottle.
- **Prep 70% ethanol**. 1400 µL per 1.5mL microcentrifuge tube. 980 µL ethyl alcohol + 420 µL sterile water.
- Perform all steps of procedure at room temperature and work quickly.
- Perform all centrifugation steps at 20-25°C
- Label QIAshredder spin column placed in 2 mL collection tube (purple columns)
- Label and cap gDNA Eliminator spin column placed in a 2 mL collection tube (clear columns)
- Label RNeasy spin column placed in a 2 mL collection tube (pink columns)
- Grab empty 2 mL collection tubes for step 24
- Label clear 1.5 mL microcentrifuge tubes for the final RNA sample

Protocol

1. In the cell culture hood, label 15 mL centrifuge tubes with the name of your samples.
2. Remove alginate gels from the incubator and place in the hood.
3. Add 1 mL of 100mM EDTA to each gel.
4. Allow the gels to dissociate in the $CaCl_2$. Pipetting of the gel/$CaCl_2$ solution may be necessary to break up the gel. Pipet the gel/$CaCl_2$ solution into the respective 15 mL centrifuge tube. Do this for all the gels, changing the pipet tip between each sample.
5. Centrifuge the gel/$CaCl_2$ solution at 500 G for 2 mins.
6. Remove the supernatant being careful not to disrupt the cell pellet. Add 1 mL of sterile 1X PBS to the cell pellet, wash the cells by mixing, then centrifuge at 500 G for 2 mins.
7. Repeat step 6.
8. Bring the centrifuge tubes containing just the cell pellets to the PCR counter. Spray down with DNase/RNase Away.
9. Disrupt the cells by adding 350 µL of Buffer RLT Plus to each cell pellet.
10. Pipet each pellet to mix and pipet the lysate directly into its respective QIAshredder spin column.
11. Centrifuge for 2 min at maximum speed.
12. Transfer the homogenized lysate to the respective gDNA Eliminator spin column.
13. Centrifuge for 30 sec at max speed.

14. Discard the column, and save the flow-through.
15. Add 350 µL of 70% ethanol to the flow through, and mix well by pipetting.
16. Transfer the 700 µL of the sample, to the respective RNeasy spin column. Close the lid gently.
17. Centrifuge for 15 sec at max speed. Discard the flow-through and reattach the column to the collection tube.
18. Add 700 µL Buffer RW1 to the RNeasy spin column. Close the lid gently.
19. Centrifuge for 15 sec at max speed to wash the spin column membrane. Discard the flow-through and reattach the column to the collection tube.
20. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently.
21. Centrifuge for 15 sec at max speed to wash the spin column membrane. Discard the flow-through and reattach the column to the collection tube.
22. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently.
23. Centrifuge for 2 mins at max speed to wash the spin column membrane.
24. Place each RNeasy spin column in new 2 mL collection tubes and discard the old collection tubes with the flow through.
25. Centrifuge at max speed for 1 min.
26. Place the RNeasy spin column in the final labeled 1.5 mL centrifuge tube.
27. Add 30-50 µL of sterial RNase-free water **directly** to the spin column membrane. Close the lid gently.
28. Centrifuge at max speed for 1 min to elute the RNA.
29. Trash the columns and place samples in a box in the -80°C.