8-2023

Investigation of possible inflammatory response after ultrasound and microbubble procedure in the spinal cord.

Jenna Wilkerson
University of Louisville

Investigation of Possible Inflammatory Response After Ultrasound and Microbubble Procedure in the Spinal Cord

by

Jenna N. Wilkerson

B.S. Bioengineering 2022

University of Louisville

Submitted to the Faculty of the Department of Bioengineering

in partial fulfillment of

BE 697 – Master of Engineering

August 8th, 2023

Investigation of Possible Inflammatory Response After Ultrasound and Microbubble Procedure in the Spinal Cord

Submitted by:

Jenna N. Wilkerson

A Thesis Approved on: 8/8/2023

By the following Reading and Examination Committee:

Digitally signed by David Magnuson
Date: 2023.08.08 15:30:14 -04'00'

David S. K. Magnuson, Ph.D.
Thesis Chair

Digitally signed by Jonathan Kopechek
Date: 2023.08.09 13:32:05 -04'00'

Jonathan A. Kopechek, Ph.D.
Thesis Co-Chair

Digitally signed by Thomas J. Roussel, Jr.
Date: 2023.08.08 15:36:58 -04'00'

Thomas J. Roussel, Jr., Ph.D.
Thesis Committee Member

ACKNOWLEDGEMENTS

This Thesis is dedicated to all the people who helped me along the way as well as all laboratory animals who sacrifice their lives in order to further scientific discovery.

ABSTRACT

The purpose of this experiment is to explore the inflammatory effects of focused ultrasound paired with intravenously delivered microbubbles when used in the lumbar spinal cord. There are currently very few pharmaceuticals that can successfully enter the blood spinal cord barrier (BSCB). This technique used at the lumbar enlargement might allow targeted treatment of pathologies involving the spinal cord.

To test the possible inflammatory response of this technique, an animal experiment was performed. 15 animals were given a T12 laminectomy and allowed to fully recover. Once recovered, all animals received FUS+MB at L2 and were euthanized in groups of five at three different time points. The spinal cord segments were dissected and used for quantitative polymerase chain reaction (qPCR). 10 different inflammatory primers were used to explore different types of inflammatory cells that may have been active at the time the animals were euthanized.

We found that there is minimal change in the qPCR data suggesting that there is little to no inflammatory response elicited by this procedure. Although these results are preliminary, and more tests must be done to investigate the full effects of this technique, this study provides a promising method to temporarily permeabilize the BSCB.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Spinal cord injury (SCI) affects 12,000 new people each year in the United States.[1] The level of impairment a patient has post-SCI varies depending on which spinal cord segments are affected and the severity of the injury.[2] Levels of disability include incomplete tetraplegia, complete paraplegia, incomplete paraplegia, and complete tetraplegia. Currently there is no cure for SCI, but most SCI patients undergo physical and occupational therapies which can mitigate functional impairments but do not attempt to cure SCI. Although oral medications are also used to manage symptoms such as neuropathic pain, erectile dysfunction, bowel dysfunction, bladder dysfunction, and spasticity, there is currently only one drug approved by the FDA for use in SCI.[3-6]

1.1 Pharmaceutical Limitations for SCI Symptom Treatment

A large reason the number of approved pharmaceuticals is so low is in part due to the blood spinal cord barrier (BSCB). The rate at which medications cross the BSCB is determined by drug molecular weight as well as the hydrophobicity therefore many drugs do not have the correct molecular weight and/or hydrophobicity to successfully diffuse into the spinal cord parenchyma.[7] Temporarily opening the BSCB could allow pharmaceuticals of almost any size or hydrophobicity to enter the spinal cord.

1.2 Crossing the Blood Spinal Cord Barrier

One possible approach to doing this is using focused ultrasound paired with intravenous microbubbles (FUS+MB). Using this approach, current studies in the Magnuson laboratory have targeted the lumbar spinal cord as it is crucial for locomotion and therefore an important therapeutic target following spinal cord injury.[8] For clinical translation of this technique, it is vital to understand potential side effects (such as inflammation). Although inflammation caused by this technique has been studied in the brain, the potential inflammatory response elicited in the spinal cord is unknown, and prior to clinical translation any potential inflammatory effects must be thoroughly studied in the spinal cord.[9]

1.3 Project Specific Aim

This study explores ten different inflammatory markers at three different timepoints following FUS+MB to assess the inflammatory response following this technique in the lumbar spinal cord. We hypothesized that FUS+MB would produce inflammation in the lumbar spinal cord beginning within an hour and persist for at least 48 hours.

II. BACKGROUND

FUS+MB is a potential solution to allow non-invasive delivery of pharmaceuticals to the spinal cord parenchyma, that otherwise would not cross the BSCB, however, prior to clinical use potential negative effects must be thoroughly tested. One concern is that this procedure may elicit localized inflammation in the target tissue/area.[9]

2.1. The Blood Spinal Cord Barrier

The blood spinal cord barrier (BSCB) is a semipermeable structure that is composed of tight junctions between endothelial cells surrounded by astrocytes encapsulating the blood vessels of the central nervous system (CNS)[10] which can be seen in Figure 1.[11] The BSCB consists of several cells including

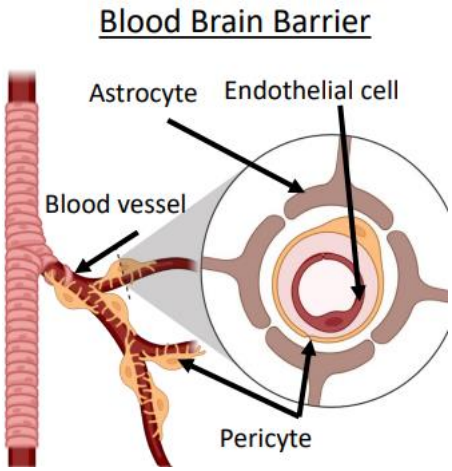

Figure 1: The Blood Brain Barrier (From BioRender.com)

endothelial cells, pericytes, and astrocytes which all provide structure and the selective permeability that the blood spinal cord barrier possesses. This barrier is an important anatomical structure that prohibits unwanted molecules such as viruses and bacteria from entering the brain and spinal cord.[12]

2.2. Opening the Blood Spinal Cord Barrier

The BSCB can be temporarily opened using FUS+MB which consists of microbubbles made with a lipid monolayer filled with perfluorocarbon gas. These bubbles are injected intravenously and allowed to circulate in the blood stream. Once in the blood stream, ultrasonic waves at a specific frequency can be used to oscillate the bubbles causing the tight junctions of the BSCB to briefly separate allowing large molecules to enter the parenchyma (Figure 2).[9] Preliminary data indicates that a 2.5% (v/v) microbubble dose and a 3.8 MPa acoustic pressure permeabilize the BSCB spanning 2-3 lumbar spinal cord segments, while

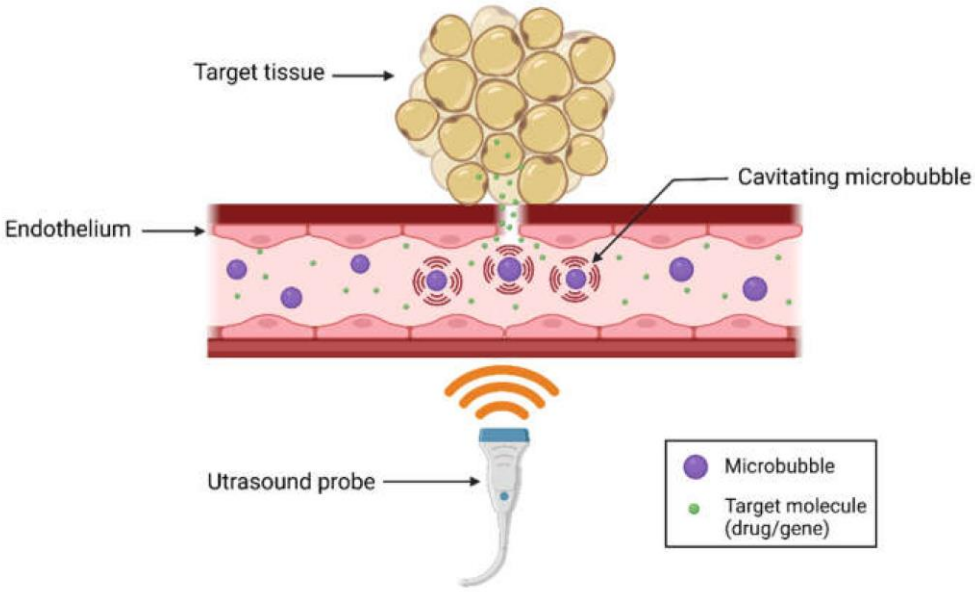

Figure 2: Focused ultrasound and microbubbles used to permeabilize the blood spinal cord barrier[30]

causing minimal histopathology at the target site (Figure 3). This technique has been shown to cause temporary inflammation in the brain in the absence of histopathology, but it is currently unknown if it causes inflammation in the spinal cord or how long it persists.

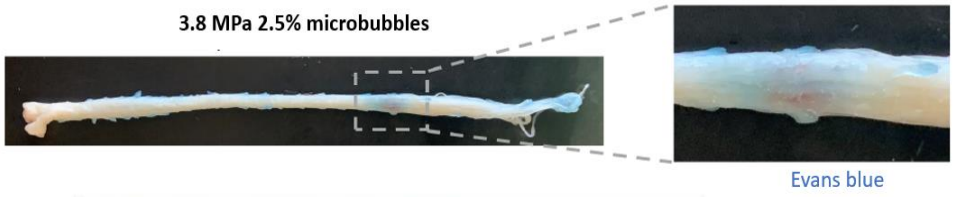

Figure 3: Evidence of spinal cord permeabilization with 3.8Mpa ultrasound pressure and 2.5% (v/v) microbubbles dose shown by Evans blue dye within the cord.[31]

2.3. Sterile vs. Non-Sterile Inflammation

For interpretation of current findings, it is important to understand the differences between sterile and non-sterile inflammation. Non-sterile inflammation occurs when a pathogen enters the body and excretes harmful cytokines, chemokines, or trophic factors (CCTFs).[13] However, sterile inflammation occurs in the absence of any pathogen or microorganism and is instead triggered by trauma or a chemically induced injury.[14] Both types of inflammation can be studied by measuring the amount of pro-inflammatory cytokines that are produced as well as the amount of inflammatory cells that are recruited to the area.

2.4. Microglia

Microglia are found throughout the CNS and are involved in immune surveillance, inflammation, and neuroinflammatory responses. These cells are particularly concentrated at/near areas of injury or infection and produce and

release various immune mediators. These mediators can have both protective and detrimental effects that are context dependent.

2.5. Quantifying the Sterile Immune Response

To quantify the sterile immune response potentially caused by FUS+MB, polymerase chain reaction (qPCR) was used. The primers selected for this study were TNF-α, CHOP, GFAP, IBA1, IL-1α, IL-1β, NF-κB, IL-6, ICAM, and CD34. General functions of these primers are summarized in Table 1.

Table 1: All qPCR markers used and a short summary of their indications.

qPCR Marker	Indication
TNF-α	Proinflammatory cytokine expressed by macrophages and monocytes.
CHOP	Transcription factor involved in ER stress response.
GFAP	An intermediate filament protein expressed in astrocytes.
IBA1	A protein used to evaluate the activation of microglia.
IL-1α	A pro-inflammatory cytokine that is involved in tissue damage.
IL-1β	A pro-inflammatory cytokine involved in immune regulation.
NF-κB	A transcription factor that plays a role in chronic inflammation.
IL-6	A protein that acts as a signaling molecule to regulate immune response.
ICAM	Intracellular adhesion molecule expressed on endothelial cells controlling leukocyte access to the BSCB.
CD34	Transmembrane glycoprotein that blocks cell adhesion in endothelial cells.

2.5.1 Tumor Necrosis Factor Alpha

TNF-α (Tumor Necrosis Factor Alpha) is a pro-inflammatory cytokine involved in the immune response and inflammatory regulation and is involved in numerous physiological and pathological processes, including host defense,

tissue homeostasis, and the pathogenesis of inflammatory diseases. Elevated expression of TNF-α is associated with conditions such as autoimmune disorders, chronic inflammation, and certain cancers. TNF-α is often produced by macrophages which are large phagocytic cells that exist in all tissues of the body and engulf/destroy pathogens.[15, 16]

2.5.2 C/EBP Homologous Protein

CHOP (C/EBP Homologous Protein), also known as DDIT3, is a transcription factor involved in endoplasmic reticulum (ER) stress response and is induced under conditions of cellular stress, such as nutrient deprivation, hypoxia, and accumulation of misfolded proteins. CHOP activation leads to apoptotic cell death or cellular adaptation to the stress conditions. qPCR analysis of CHOP expression can provide insights into ER stress processes, such as unfolded protein response (UPR) and apoptosis.

2.5.3 Glial Fibrillary Acidic Protein

GFAP (Glial Fibrillary Acidic Protein) is an intermediate filament protein predominantly expressed in astrocytes. qPCR analysis of GFAP expression helps quantify the extent of astrocyte activation in response to brain injury, neurodegenerative diseases, or other neurological disorders.[17]

2.5.4 Ionized Calcium-Binding Adapter Molecule 1

Ionized Calcium-Binding Adapter Molecule 1 (IBA1) is a calcium-binding protein, primarily expressed in microglia and is used to evaluate microglial activation.

2.5.5 Interleukin 1 Alpha

Interleukin 1 Alpha (IL-1α) is a pro-inflammatory cytokine involved in immune responses and inflammation regulation. IL-1α plays a role in various physiological and pathological processes, including infection, tissue damage, and autoimmune diseases.[18]

2.5.6 Interleukin 1 Beta

Interleukin 1 Beta (IL-1β) is another pro-inflammatory cytokine involved in immune responses and inflammation regulation, IL-1β has similar functions to and is often co-expressed with IL-1α.

2.5.7 Nuclear Factor Kappa B

Nuclear Factor Kappa B (NF-κB) is a transcription factor that is involved in the regulation of immune responses, inflammation, and cell survival. NF-κB controls the expression of numerous genes involved in immune and inflammatory pathways, and dysregulation is associated with various diseases, including cancer, autoimmune disorders, and chronic inflammation.

2.5.8 Interleukin 6

Interleukin 6 (IL-6) is a protein that acts as a signaling molecule to regulate immune cell function during infections, the regulation of inflammation, and the promotion of the acute-phase response.

2.5.9 Intercellular adhesion molecule 1

Intercellular adhesion molecule 1 (ICAM) is a cellular adhesion molecule located on the outside of endothelial cells. This molecule is involved in the movement of leukocytes into the spinal cord parenchyma.[19]

2.5.10 CD34

CD34 is a sialomucin that blocks cell adhesion and is typically found on endothelial cells.[20]

III. METHODS

3.1. Animal Model

For this study, N=15 Sprague Dawley rats were split into three groups of five. Animals were housed two per cage, given access to food and water as needed, and placed on a 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Care and Use and Institutional Biosafety Committee at the University of Louisville.

3.2. Animal Surgical Technique and Laminectomy

Animals were handled for ten minutes once a day for one week prior to any procedures. For surgery, animals were anesthetized using isoflurane and the backs of the animals were shaved and disinfected. A sagittal incision was made across the mid back from T10-T13. A T12 laminectomy (a procedure in which one spinal cord lamina was removed to expose the spinal cord) was performed to expose the L2 spinal cord. Following laminectomy, muscle was sutured closed, skin stapled, and bacitracin applied to the surgical site.

3.3. Animal Recovery and Anesthetization

Animals were given 0.25 mL meloxicam once per day for 72 hours post laminectomy and 0.35 mL buprenorphine once per day for 24 hours post laminectomy. Staples were removed seven days post-surgery once surgical sites

were fully healed. Three weeks following laminectomy, animals were anesthetized using isoflurane for FUS+MB procedures.

3.4. FUS+MB Procedures

Once a stable plane of anesthesia was achieved, the backs of the animals were shaved and ultrasound gel liberally applied. Ultrasound imaging was used to confirm correct placement of the transducer at the level of the laminectomy. Once confirmed, an injection of 0.15 mL 2.5% (v/v) microbubbles into the tail vein followed by a saline flush and ultrasonicated at 3.8 MPa for two minutes in groups of 5. To evaluate inflammation tissue was harvested 6-, 24-, and 72-hours post FUS+MB (Full experimental design flow shown in Figure 4). Animals were euthanized using a cocktail of ketamine, xylazine, and acepromazine (40, 2.5, and 1 mg/kg, i.p.). Once toe pinch withdraw was absent, animals were transcardially perfused with phosphate-buffered saline (pH 7.4).

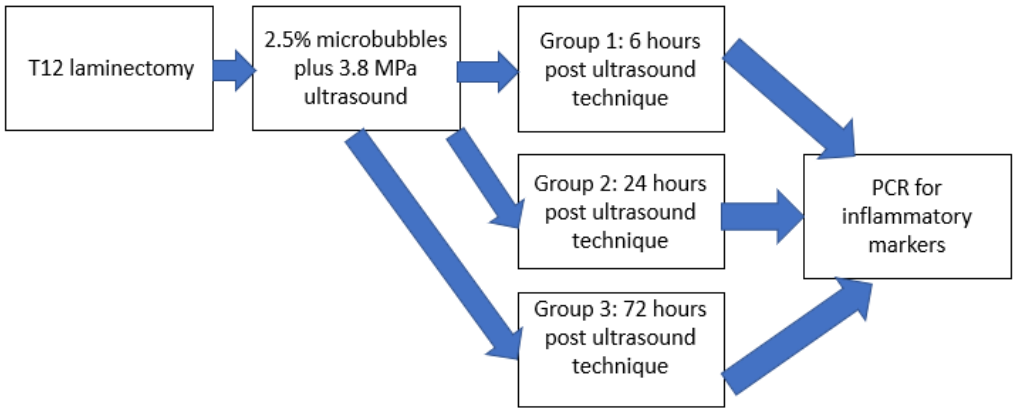

Figure 4: Experimental design

3.5. Spinal Cord Harvesting

Spinal cords were harvested, each spinal enlargement was allocated into a tube and flash frozen in liquid nitrogen. Spinal cord segments from each

enlargement were weighed (0.04 grams each) to obtain similar portions of the cord. The portions were then homogenized using VWR cordless pestle motor (47747-370, VWR, Radnor PA).

3.6. RNA Isolation and Analysis

RNA was isolated using TRIzol (15596026, ThermoFisher, Waltham, MA) then analyzed using the Nanodrop spectrophotometer (ND2000USCAN, ThermoFisher, Waltham, MA). Each sample then went through cDNA synthesis using the high-capacity cDNA reverse transcriptase kit (4368814, ThermoFisher, Waltham, MA). Following this, a quantitative polymerase chain reaction was performed using 384 well PCR plates (89218-294, VWR, Radnor PA), TaqMan 2X universal PCR master mix (434437, ThermoFisher, Watham, MA), and custom TaqMan gene expression assays (4331182, ThermoFisher, Watham, MA). All primers were compared to the house primer GAPDH (4352338, ThermoFisher, Watham, MA). The plates were analyzed in the ViiA 7 real-time PCR system (4453545, ThermoFisher, Watham, MA).

3.7. Statistical Analysis

A power analysis was executed to calculate the sample size needed for each group using a power value of 0.8 and an alpha value of .05. SSPS (IBM, Armonk, New York) was used to perform a repeated measures analysis of variance (ANOVA) and when appropriate paired post hoc T tests were used to compare the fold change (the ratio of the house keeping primer, GAPDH, measurement and the primer of interest) for each primer was measured at each timepoint as well as in the cervical and lumbar regions. Delta-delta Ct values

were calculated for each primer to compare the marker of interest to the house keeping primer. Data points with standard deviations > 2.5 from the mean for each marker and time point were considered outliers and were removed from the data set(s).

IV. RESULTS

To assess the inflammatory response in the spinal cord at L2, a portion of lumbar cord and a portion of cervical cord were taken from each animal.

Figure 5 shows TNF-α fold change between cervical and lumbar segments of the spinal cord over three timepoints. At 6 hours post FUS+MB, fold change was significantly greater when compared to 72 hours post FUS+MB. In the same figure, at 6 hours post FUS+MB the fold change was almost significantly increased compared to the 24-hour timepoint.

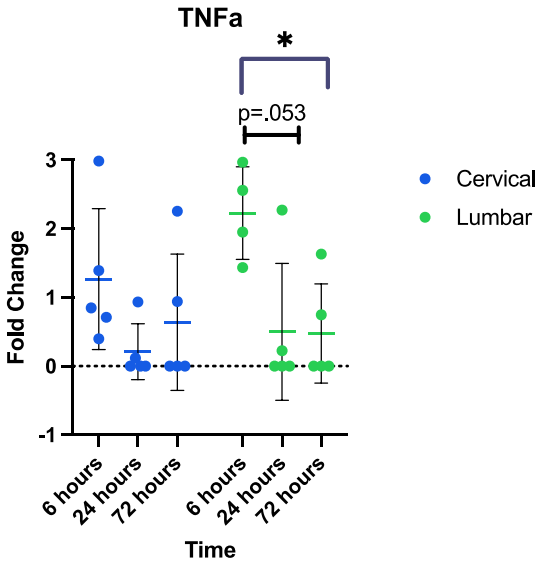

Figure 5: TNFa Results.

Figure 6, depicting the results for NF-κB, shows no significant changes in cervical or lumbar portions across all timepoints.

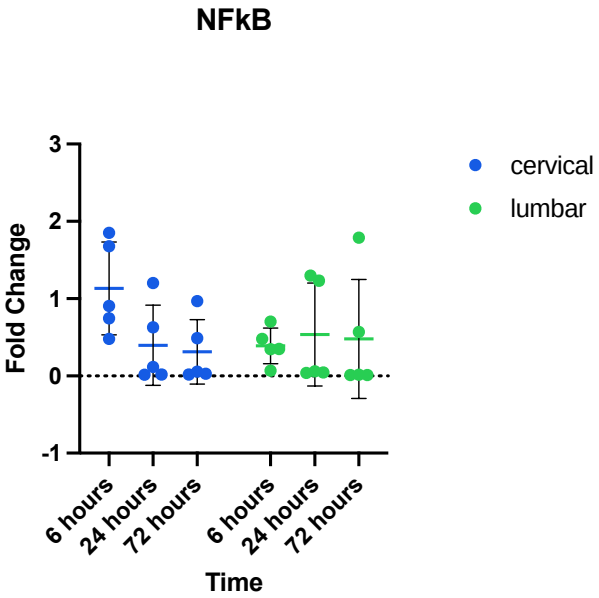

Figure 6: NF-κB Results

Figure 7, depicting IL-6, shows no significant changes in cervical or lumbar portions across all timepoints.

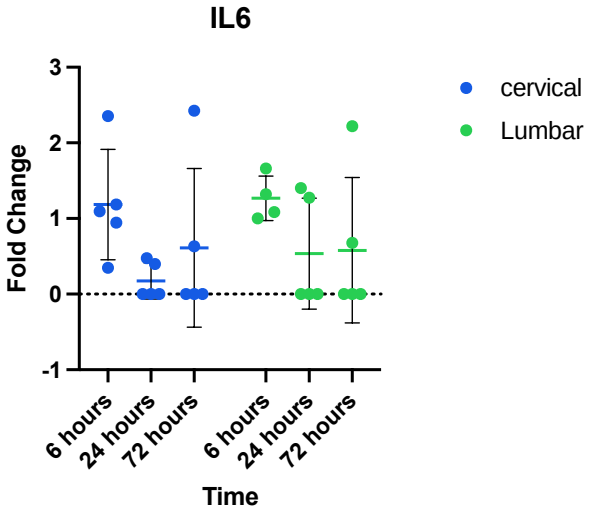

Figure 7: IL6 Results

Figure 8, depicting IL-1β, shows no significant changes in cervical or lumbar portions at all timepoints.

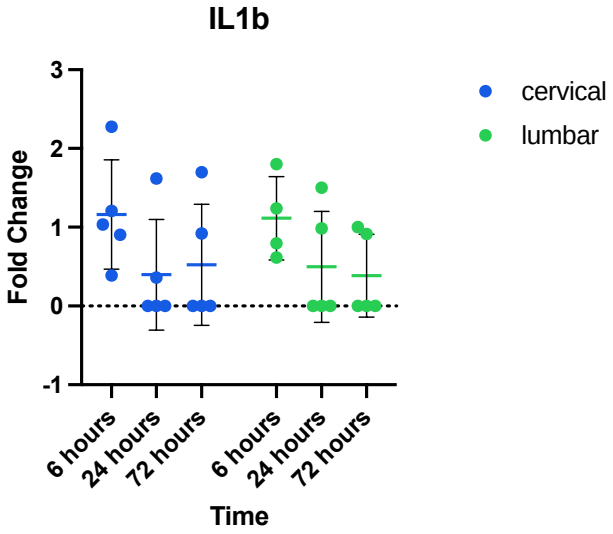

Figure 8: IL1b Results

Figure 9 depicting, IL-1A, shows no significant changes in cervical or lumbar portions at all timepoints.

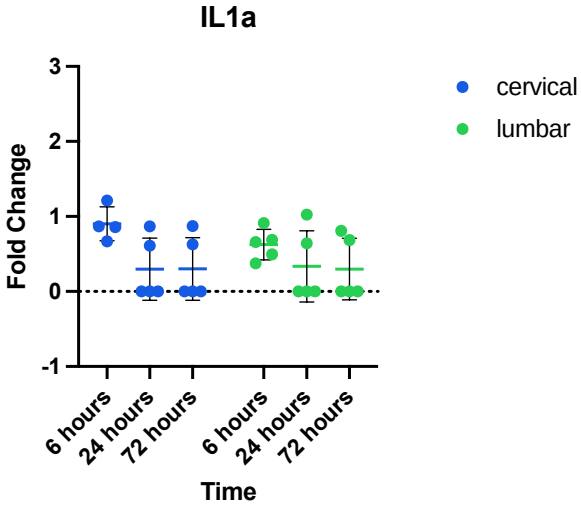

Figure 9: IL1a Results

Figure 10, depicting ICAM, shows no significant changes in cervical or lumbar portions at all timepoints.

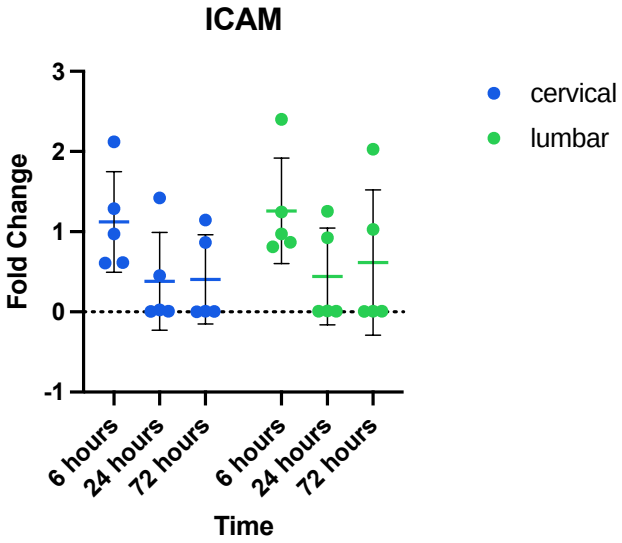

Figure 10: ICAM Results

Figure 11 shows an almost significant increase at 6 hours post FUS+MB compared with 24 hours in the cervical region as well as an almost significant increase in the lumbar region at 6 hours post FUS+MB compared to 72 hours.

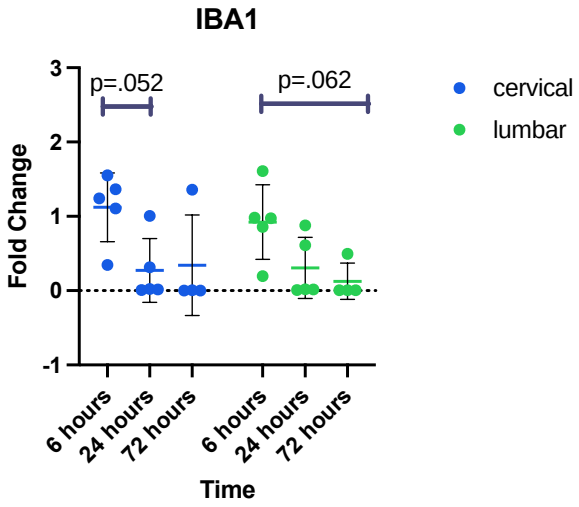

Figure 11: IBA1 Results

Figure 12, depicting GFAP, shows no significant changes in cervical or lumbar portions at all timepoints.

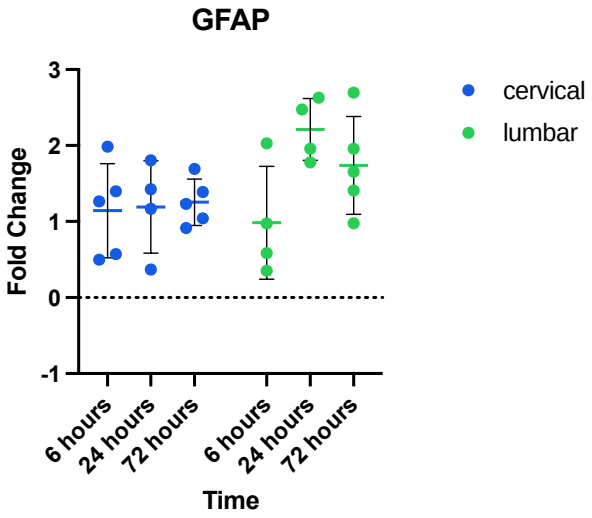

Figure 12: GFAP Results

Figure 13, the results for CHOP, shows an almost statistically significant fold change increase at 6 hours post FUS+MB when compared to 24 hours in the cervical region.

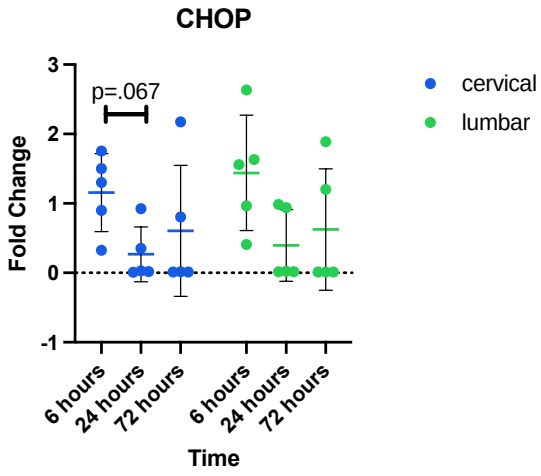

Figure 13: CHOP Results

Figure 14, depicting CD34, shows no significant changes in cervical or lumbar portions at all timepoints.

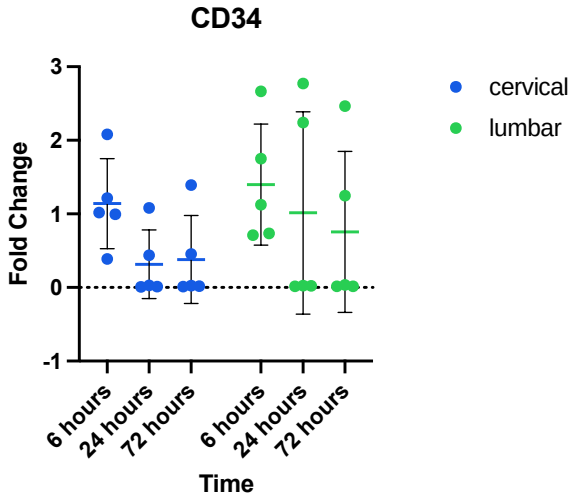

Figure 14: CD34 Results

From these results, it was found that TNF-α was the only significant marker in the lumbar region (TNF-α: 6 hr vs. 72 hr: p = 0.022800). TNF-α also trended downwards between 6 and 24 hours (p =.053). IBA1 also showed a trend in both the cervical and lumbar region of the cord (IBA1: 6hr vs. 24hr p =.052 and 6hr vs. 72hr p =.062 respectively). CHOP also trends downwards in the cervical region (CHOP: 6hr vs. 24hr p = .067).

V. Discussion

Preliminary testing suggested that FUS+MB could be used to permeabilize the BSCB in the lumbar spinal cord which would likely allow for pharmaceuticals to enter the parenchyma. However, before this can be used clinically, potential deleterious effects must be thoroughly studied. The inflammatory response has been studied in the brain but has yet to be evaluated in the spinal cord.[9] To explore possible inflammatory effects in the spinal cord, the exchange of cytokines between the brain and blood tissues were investigated.[21] We hypothesized that FUS+MB would produce inflammation in the lumbar spinal cord beginning within an hour and persist for at least 48 hours. We found that a majority of the inflammatory markers evaluated did not increase at any of the timepoints evaluated. TNF-α peaked at 5 minutes and 6 hours after FUS+MB when this technique was used on the brain[9] and when measured in the spinal cord, the fold change also peaked at 6 hours. However, by 72 hours post FUS+MB, TNF-α levels decreased. While the change between the 6 and 24-hour timepoints was not significant, a small sample size was used for the experiments. If a larger sample size was used, TNF-α may have been significantly different between the -6 and 24-hour points. This increase in TNF-α may result from the disruption of cells that make up the BSCB. TNF-α is a cytokine that is typically released when there is a disruption in tissue homeostasis therefore the disruption

of the endothelial cells could be causing this increase. When measured in the brain, TNF-α was found to be significant through 24 hours.[9] This could suggest that the disruption of endothelial cells in the spinal cord is more short-term than when this technique is used on brain tissue.

When FUS+MB is used in the brain, cytokines associated with NF-κB pathways were upregulated at 6 hours post procedure.[9] However, in the spinal cord, the NF-κB pathway was not upregulated at any timepoint evaluated in this study. This is a surprising finding since the NF-κB pathway activates many cytokines, chemokines, and adhesion molecules including, but not limited to, IL-1α, IL-6, TNF-α, and ICAM.[22] One reason for this difference in the spinal cord might be because of the differences in the tissues. The spinal cord contains grey and white matter like the brain but unlike the brain the white matter engulfs the grey matter.[23] The white matter consists of fewer cell bodies than the grey matter and has axons as well which are myelinated.[23] The myelin sheath is made of fat and helps conduct electricity through the axons.[23] This fat makes the mechanical and electrical properties of the white matter different than the grey matter.[24] This might account for some of the differences in response to FUS+MB in the spinal cord when compared to the brain.

IL-6 did not show a significant change in this study. This is surprising considering its relationship with TNF-α increase post FUS+MB. These molecules are endogenous pyrogens which are typically expressed together.[25] Despite this, when a similar study was performed on the brain, IL-6 only increased up to 30

minutes after the technique was performed.[9] This data is harmonious with the data presented here since the first timepoint tested was 6 hours post FUS+MB.

Previous studies of FUS+MB used on the brain suggest a significant increase of IL-1β in the brain at 2-, 6-, and 12-hours post procedure however here we report no increase at any of the timepoints evaluated.[9] After an injury in the spinal cord IL-1β increases immediately and has been shown to continue to be elevated for 6 days.[26] Since no injury is resulting from FUS+MB, there might be a peak early on and a decrease in IL-1β expression by 6 hours. Along with IL-1β, IL-1α showed an increase at 2-, 6-, and 12-hours post FUS+MB in the brain.[9] The reason no statistical difference is seen in this experiment is likely the same reason no difference is seen in IL-1β.

ICAM levels remained the same after FUS+MB in this experiment. When FUS+MB procedures were used in the brain, ICAM expression increased at 12- and 24-hours post procedure.[9] This may also be a result of small group size or there could be no leukocyte movement into the spinal cord parenchyma at the timepoints tested. IBA1 trended towards an increase in the cervical region 6 hours post FUS+MB compared to 24 hours as well as 6 hours post FUS+MB compared to 72 hours in the lumbar region. In previous studies done on brain tissue IBA1 is shown to peak at 6 hours as well.[9]

Similarly, GFAP expression was not found to be significantly different post FUS+MB in the spinal cord. Previous studies using FUS+MB procedures on the brain found an increase in GFAP expression at 6- and 24-hours post procedure.[9] In a similar study, GFAP was significantly higher at 7 weeks post treatment.[27]

Pulsed focused ultrasound (pFUS), delivered in 10-ms bursts at 0.3 MPa, was used in the investigations in the brain that demonstrated expression at 6 and 24 hours after ultrasound.[9] When we conducted these studies on the spinal cord, ultrasound was delivered in similarly timed pulses for 2 minutes but differed in terms of ultrasonic pressure. These changes could contribute to the lack of significant changes. Microbubbles similar in structure to the bubbles used in the spinal cord were used in the brain but the dose was not specified.[9] Time constraints may have also been a reason for this lack of expression and using a later timepoint might reveal some disruption in the astroglia cells or cytokines that may indicate inflammation.

CHOP levels trended towards an increase (but were not statistically different), but only in the cervical spinal cord at 6 hours post FUS+MB. One might expect changes to be larger in the lumbar region since that was the targeted area. One reason that CHOP might be upregulated in the cervical spinal cord is due to the presence of long ascending propriospinal neurons (LAPNs). LAPNs have cell bodies in the lumbar enlargement (the target of FUS+MB) and axons that ascend to synapse in the cervical enlargement. Causing a disruption to the lumbar spinal cord where the LAPN cell body is located could have caused an immune response at the cervical terminal point of the neuron. Similarly, long descending propriospinal neurons (LDPNs) have a cell body in the cervical spinal cord and terminate at the lumbar spinal cord. A disruption in the lumbar spinal cord might cause an immune response in the cell body. This trend might also be the result of a systemic immune response, though this is unlikely. CD34 is shown

to have a negative correlation with spinal cord barrier permeability and suggests that the BSCB is no longer permeable at 6 hours post procedure.[28]

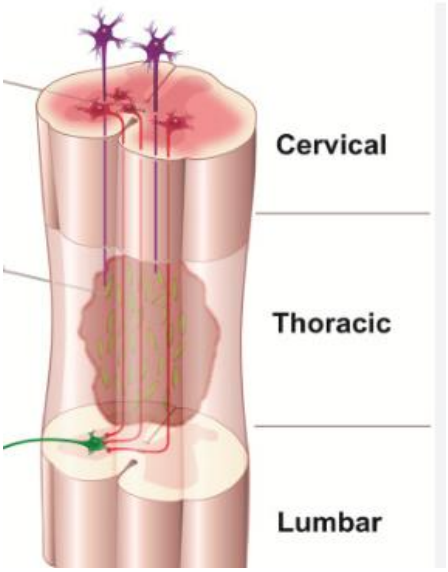

Figure 15: LAPNs have cell bodies in the lumbar enlargement and axons that ascend to synapse in the cervical enlargement.[32]

Another possible reason that these inflammatory markers were not amplified is the variability of FUS+MB and the tissue isolation techniques. When isolating the tissue for RNA synthesis the point of ultrasound was unclear, therefore, although all sections of cord were of uniform weight, some sections might be above or below the ultrasound target sight. This might result in differences in inflammatory marker increase between animals. Another possible reason for variability is the differences in individual animals' health. Although all animals were kept in the same housing arrangement, outside factors such as regular cage cleanings, feedings, and social treatment by other rats might have cause some stress on the animals possibly causing an immune response.

For future studies, more control groups must be added to get a complete understanding of the effects of this technique. It is important to understand the response to the laminectomy alone as well as the microbubble injection without ultrasound waves applied. These controls will allow exploration of baseline inflammation that might be occurring. Along with the added controls, other measurements should be used to completely understand the immune response. qPCR alone only shows the presence of RNA in the tissue and does not directly measure specific chemokines, cytokines, and trophic factors (CCTFs) that are present. These CCTFs might be present long after the RNA that codes for them is being produced. A test like ELISA can be used to directly measure CCTFs in addition to qPCR to understand the time course of the immune response initiated by FUS+MB. Along with qPCR and ELISA, immunohistochemistry can be used to look at specific immune cells that might be present after FUS+MB. All these tests combined would give a comprehensive overview of CCTFs and immune cells allowing for a more definitive conclusion on the immune response elicited by FUS+MB.

There are several clinical applications for this procedure with the main one being focal delivery of pharmaceuticals into the spinal cord parenchyma. Currently, more than 98% of pharmaceuticals cannot cross the blood spinal cord or brain barrier.[29] Out of the less than 2% that can, only one is FDA approved for use in spinal cord injury patients.[3] This technique may not only allow a pharmaceutical to cross the BSCB, but also allows for the targeting of a specific section of the spinal cord with the drug. This would be extremely prudent for

targeting the areas responsible for locomotion in the spinal cord without allowing the pharmaceutical into the brain. This specificity could also allow targeted pharmaceutical delivery to intramedullary tumors or be used to target only the spinal cord in amyotrophic lateral sclerosis (ALS) treatments.

VI. Conclusion

We hypothesized that the inflammatory markers would be elevated for at least 48 hours after FUS+MB. Surprisingly, very few markers increase at 6-, 24-, and 72-hours post FUS+MB. The only marker that increased was TNF-α. This suggests that FUS+MB targeting the lumbar enlargement results in minimal inflammatory response. Although not statistically significant, CHOP and IBA1 showed trends suggesting some sort of inflammatory response at 6 hours post FUS+MB. Future studies will need to evaluate similar responses at various times post-SCI if FUS+MB is going to be translated to the clinic. However, this technique provides a promising method for focal BSCB permeabilization and non-invasive drug delivery to the spinal cord.

REFERENCES

1. Ma, V.Y., L. Chan, and K.J. Carruthers, Incidence, prevalence, costs, and impact on disability of common conditions requiring rehabilitation in the United States: stroke, spinal cord injury, traumatic brain injury, multiple sclerosis, osteoarthritis, rheumatoid arthritis, limb loss, and back pain. Archives of physical medicine and rehabilitation, 2014. **95**(5): p. 986-995. e1.
2. Fouad, K., et al., *The neuroanatomical–functional paradox in spinal cord injury.* Nature Reviews Neurology, 2021. **17**(1): p. 53-62.
3. Dalal, K.L., E.R. Felix, and D.D. Cardenas, *Pregabalin for the management of neuropathic pain in spinal cord injury.* Pain Management, 2013. **3**(5): p. 359-367.
4. Johns, J., et al., Management of neurogenic bowel dysfunction in adults after spinal cord injury: clinical practice guideline for health care providers. The Journal of Spinal Cord Medicine, 2021. **44**(3): p. 442-510.
5. Benevento, B.T. and M.L. Sipski, Neurogenic bladder, neurogenic bowel, and sexual dysfunction in people with spinal cord injury. Physical therapy, 2002. **82**(6): p. 601-612.
6. Burchiel, K.J. and F.P. Hsu, Pain and spasticity after spinal cord injury: mechanisms and treatment. Spine, 2001. **26**(24S): p. S146-S160.
7. Pardridge, W.M., *Drug transport across the blood–brain barrier.* Journal of cerebral blood flow & metabolism, 2012. **32**(11): p. 1959-1972.
8. Hubli, M. and V. Dietz, *The physiological basis of neurorehabilitation-locomotor training after spinal cord injury.* Journal of neuroengineering and rehabilitation, 2013. **10**(1): p. 1-8.
9. Kovacs, Z.I., et al., *Disrupting the blood–brain barrier by focused ultrasound induces sterile inflammation.* Proceedings of the National Academy of Sciences, 2017. **114**(1): p. E75-E84.
10. Serlin, Y., et al. Anatomy and physiology of the blood–brain barrier. in Seminars in cell & developmental biology. 2015. Elsevier.
11. *The Blood Brain Barrier.* [Reprinted from "Brain Vascular system"] 2023; Available from: https://app.biorender.com/biorender-templates.
12. Bernacki, J., et al., *Physiology and pharmacological role of the blood-brain barrier.* Pharmacol Rep, 2008. **60**(5): p. 600-622.
13. Sherwood, E.R. and T. Toliver-Kinsky, *Mechanisms of the inflammatory response.* Best Practice & Research Clinical Anaesthesiology, 2004. **18**(3): p. 385-405.

14. Chen, G.Y. and G. Nuñez, *Sterile inflammation: sensing and reacting to damage.* Nature Reviews Immunology, 2010. **10**(12): p. 826-837.
15. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-455.
16. Riches, D.W., E.D. Chan, and B.W. Winston, *TNF-α-induced regulation and signalling in macrophages.* Immunobiology, 1996. **195**(4-5): p. 477-490.
17. Jurga, A.M., et al., *Beyond the GFAP-astrocyte protein markers in the brain.* Biomolecules, 2021. **11**(9): p. 1361.
18. Raivich, G., et al., Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain research reviews, 1999. **30**(1): p. 77-105.
19. Dietrich, J.-B., The adhesion molecule ICAM-1 and its regulation in relation with the blood–brain barrier. Journal of neuroimmunology, 2002. **128**(1-2): p. 58-68.
20. Siemerink, M.J., et al., CD34 marks angiogenic tip cells in human vascular endothelial cell cultures. Angiogenesis, 2012. **15**: p. 151-163.
21. Chen, S., et al., A review of bioeffects induced by focused ultrasound combined with microbubbles on the neurovascular unit. Journal of Cerebral Blood Flow & Metabolism, 2022. **42**(1): p. 3-26.
22. Liu, T., et al., *NF-κB signaling in inflammation.* Signal transduction and targeted therapy, 2017. **2**(1): p. 1-9.
23. Nógrádi, A., A. Nógrádi, and G. Vrbová, *Anatomy and physiology of the spinal cord.* Transplantation of neural tissue into the spinal cord, 2006: p. 1-23.
24. Palombo, M., et al., Abundance of cell bodies can explain the stick model's failure in grey matter at high bvalue. 2018.
25. Muylle, L., et al., Increased tumor necrosis factor alpha (TNF alpha), interleukin 1, and interleukin 6 (IL-6) levels in the plasma of stored platelet concentrates: relationship between TNF alpha and IL-6 levels and febrile transfusion reactions. Transfusion, 1993. **33**(3): p. 195-199.
26. Choi, B.M., et al., The time-course and RNA interference of TNF-α, IL-6, and IL-1β expression on neuropathic pain induced by L5 spinal nerve transection in rats. Korean J Anesthesiol, 2015. **68**(2): p. 159-169.
27. Kovacs, Z.I., et al., MRI and histological evaluation of pulsed focused ultrasound and microbubbles treatment effects in the brain. Theranostics, 2018. **8**(17): p. 4837.
28. Jin, X., et al., Negative Correlation of CD 34+ Cells with Blood–Brain Barrier Permeability following Traumatic Brain Injury in a Rat Model. Microcirculation, 2014. **21**(8): p. 696-702.
29. Pardridge, W.M., The blood-brain barrier: bottleneck in brain drug development. NeuroRx, 2005. **2**: p. 3-14.
30. Sanwal, R., Joshi, K., Ditmans, M., Tsai, S. S. H., & Lee, W. L. (2021). Ultrasound and Microbubbles for Targeted Drug Delivery to the Lung Endothelium in ARDS: Cellular Mechanisms and Therapeutic

Opportunities. *Biomedicines 2021, Vol. 9, Page 803*, *9*(7), 803. https://doi.org/10.3390/BIOMEDICINES9070803

31. Wilkerson, J. N., Brown, B. L., Whittemore, S. R., Kopechek, J. A.,; Magnuson, D. S. K.. Focused Ultrasound with Intravenous Microbubbles Disrupts the Blood Brain Barrier co-first authors Experimental Overview Thoracolumbar Vertebrae Attenuates Ultrasound Signal
32. Deng, L., Ravenscraft, B.; Xu, X. M. (2022). Exploring propriospinal neuron-mediated neural circuit plasticity using recombinant viruses after spinal cord injury. Experimental Neurology. https://doi.org/10.1016/j.expneurol.2021.113962