THE INFLUENCE OF LPS-TREATED BV2 SUPERNATANTS ON GLUTAMATE INDUCED CELL DEATH IN HT22 CELLS

by

Raleigh Robinson

Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 5, 2025

THE INFLUENCE OF LPS-TREATED BV2 SUPERNATANTS

ON GLUTAMTE INDUCED CELL DEATH

IN HT22 CELLS

Project Approved:

____

Supervising Professor: Michael Chumley, PhD
Department of Biology

____

Gary Boehm, PhD
Department of Psychology

____

Matt Chumchal, PhD
Department of Biology

ACKNOWLEDGEMENTS

I would like to thank Dr. Chumley for his role as the main supervising professor for this departmental honors research project. He has been an incredible mentor, and he has helped me navigate numerous setbacks with patience, resilience, and encouragement. I would also like to thank Dr. Boehm for his feedback in this project and leadership in the TCU Neurobiology of Aging Labs. Furthermore, I greatly appreciate the guidance of Dr. Chumchal, who has been incredibly encouraging and impactful in my work in and out of the lab over the past two years. I am also grateful to the graduate students and undergraduate students in our lab who have gone through the learning process with me and offered helpful advice as I have worked on this project. Lastly, I would like to thank TCU SERC (Science and Engineering Research Center) Grant and the National Institute of General Medical Sciences (NIH) for the generous funding we have received for this project.

ABSTRACT

Alzheimer's disease (AD) was the fifth leading cause of death in people over 65 in 2021, and an estimated 13 million Americans will have AD by 2050. AD is a neurodegenerative disease characterized by memory loss and cognitive decline due to neuronal cell death. While the exact causes are still being studied, neuroinflammation, or inflammation in the brain, is known to play a role, with microglial cells—immune cells of the brain—being a key contributor. When overactivated, microglia release excessive inflammatory molecules, which may contribute to AD progression. To investigate this, we used HT22 cells, a mouse neuronal cell, and BV2 cells, a mouse microglial cell that produces inflammatory molecules in their "conditioned" media. We treated HT22 cells with glutamate to induce cell death and exposed them to BV2-conditioned media, then measured cell survival to determine if inflammatory molecules contribute to neuronal death. Unexpectedly, we found that BV2-conditioned media reduced the toxic effects of glutamate and promoted neuron survival. These findings suggest that microglial cells may have protective as well as harmful effects in AD, highlighting the complexity of neuroinflammation in disease progression.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.. iii

ABSTRACT.. iv

INTRODUCTION.. 1

METHODS 7

RESULTS.. 11

DISCUSSION.. 14

CONCLUSION... 17

REFERENCES... 18

INTRODUCTION

Alzheimer's disease (AD) was the fifth leading cause of death in people over 65 in 2021, and it is expected that 13 million Americans will have AD by 2050 [1]. AD is a neurodegenerative disease that is characterized clinically by the onset of memory loss and cognition decline in aging populationsButterfield and Lauderback [2]. Initially, AD affects the areas of the brain involved in memory, such as the hippocampus and the entorhinal cortex. As it progresses, other regions of the brain are impacted, leading to a decline in affected individuals' ability to function properly [3].

AD is pathologically characterized by the accumulation of amyloid-beta (Aβ) plaques, neurofibrillary tau tangles, and synapse loss, which disrupts communication between neurons in the brain and leads to a widespread loss of brain function [2]. Aβ peptides take on many different forms, with beta-amyloid 42 being considered particularly toxic; it results from the cleavage of a larger protein, amyloid precursor protein (APP), by the beta-secretase enzyme [3, 4]. Aβ plaques found in the brain are known to contain high levels of calcium, copper, iron, and zinc metal ions, which are associated with oxidative stress. The binding of these metal ions promotes the aggregation of Aβ plaques by reducing the net charge of the complex and decreasing the electrostatic repulsion between two Aβ peptides, increasing their tendency to aggregate [5]. While the Aβ peptides aggregate both intracellularly and extracellularly, the intracellular aggregates are of particular interest because they are known to hinder mitochondrial function, affecting its morphology and integrity and leading to dysfunction in metabolic processes. Studies have also shown that Aβ plaques contribute to the production of reactive oxygen species (ROS), which are highly reactive oxygen-containing molecules, oxidative stress, hyperphosphorylation and accumulation of Tau, reduced energy metabolism, and provoke inflammatory responses that

lead to synaptic dysfunction and neurodegeneration. Aβ plaques thus contribute to the neuronal cell death characteristics of AD [4]. The TCU Neurobiology of Aging Lab, has historically demonstrated the link between inflammation, APP cleavage to make Aβ, and the hyperphosphorylation of Tau, as well as these processes and their connection to the cognitive dysfunction associated with Alzheimer's Disease [6, 7].

Another hallmark of AD is neurofibrillary tangles. In AD, the microtubule-associated protein tau is hyperphosphorylated at approximately nine residues compared to the normal 2–3 phosphorylations, leading to denaturation and release from microtubules. These hyperphosphorylated proteins aggregate into tau tangles and form neurofibrillary tangles [8, 9]. Such tangles disrupt the transport system in nerve cells, blocking communication between neurons and contributing to neurodegeneration and disease progression. Tau tangles may also contribute to the neuroinflammation and cell damage associated with AD [9]. Aβ plaques and Tau tangles act synergistically, with early stages of memory loss reported to be a result from their combined effects [4].

Oxidative stress and neuroinflammation are two key processes contributing to the onset and progression of AD. The term oxidative stress refers to an imbalance between the production of ROS and antioxidants [10]. ROS are highly reactive oxygen-containing free radicals. ROS, such as hydrogen peroxide and superoxide anions, form as side products of metabolisms, like oxidative phosphorylation, or come from exogenous sources, like radiation, infection, or lifestyle factors. In contrast, antioxidants, such as glutathione peroxidase and superoxide dismutase, are any enzymes or molecules that function to donate electrons to free radicals to neutralize them [11]. While ROS and antioxidants are both normally present, oxidative stress occurs when ROS levels exceed antioxidant capacity. When there are not enough antioxidants to neutralize the ROS

present, they scavenge for electrons and react with macromolecules, leading to cell damage, cell death, and ultimately disease progression [12]. For example, ROS can react with phospholipids in the cell membrane via lipid peroxidation-- a process involving the degradation of carbon-carbon bonds in lipids [13]. Lipid peroxides go on to react with and destroy proteins, compromising cell function and membrane integrity [14]. The buildup of this cellular damage eventually leads to cell death, which accumulates to tissue damage and disease [15]. While oxidative stress begins on the molecular level, it eventually effects the entire organism.

The dysregulation of free metal ions is another aspect of oxidative stress. These ions contribute to the activity of antioxidant enzymes. For example, copper ions play a role in the function of superoxide dismutase, an enzyme that neutralizes superoxide ions [16]. When dysregulated, these ions can contribute to ROS generation and cell death via ferroptosis. Ferroptosis occurs due to intracellular accumulation of iron ions, lipid peroxidation, and the reduction of glutathione levels. Ferrous ions (Fe^{2+}) catalytically decompose hydrogen peroxide (H_2O_2) into ferric ions (Fe^{3+}) and highly reactive hydroxyl radicals; this promotes ferroptosis through lipid peroxidation. Glutathione and glutathione peroxidase work in conjunction to have antioxidant activity. They neutralize lipid peroxides, being the only antioxidant capable of directly neutralizing phospholipid hydroperoxides [17].

Neuroinflammation is another process that contributes to Alzheimer's disease onset and progression, through the overactivation of the innate immune system in the CNS. Cells in the innate immune system phagocytose harmful substances, release inflammatory signals, and recruit other immune cells. Microglia, known as "guardians of the brain," survey for harmful substances, dying cells, debris, and clumps of misfolded proteins and are the primary players in neuroinflammation [18]. In a healthy brain, microglia produce anti-inflammatory cytokines and

phagocytose Aβ plaques via TAM receptors. This unique family of receptor tyrosine kinase that include Tyro3, Axl, and Mertk, regulate microglial functions like the clearance of cellular debris and apoptotic cells [19]. They also form protective barriers around senile plaques to limit their spread and mitigate tau toxicity [20]. In AD, however, microglial cells (MGCs) are overactivated by ROS, lipids released from stressed neurons, and Aβ plaques. Activated MGCs secrete proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, leading to reduced Aβ phagocytosis, activation of the complement system, neuroinflammation, and eventually neuronal cell death, which contributes to AD progression [5, 21]. Dysregulated TNF-α, in particular, induces apoptosis, leading to the neurodegeneration characteristic of AD [22]. Although inflammation is necessary for the immune response, in AD, MGC overactivation results in oxidative stress, cell damage, and cell death, even in the absence of a pathogen [23]. In this chronic state of activation, the MGCs become so overactivated that they can no longer remove Aβ but continue to release proinflammatory mediators and ROS, contributing to the oxidative stress, inflammation, and neurodegeneration characteristic of AD [24].

Additionally, previous research has demonstrated that MGCs in AD transgenic mice increase in number, a process called microgliosis, before plaque formation, and, later in disease progression, become overactivated and congregate around Aβ plaques [25]. One potential method to attenuate the role of microglia in the progress of AD is drug treatment. One study found that oral administration of Taurine, a semi-essential amino acid, reduces the overactivation of microglia in AD transgenic mice thus attenuating AD pathology [26]. Furthermore, cannabinoids were found to have a therapeutic effect on AD transgenic mice by counteracting the overproduction of TNF-α by microglia characteristic of AD while promoting the migration of microglia to Aβ plaques [27] . The combination of neuroprotective and anti-inflammatory

therapeutic effects of drugs are of interest in the prevention of AD inflammation. The potential of these compounds as therapeutic agents for AD suggests the need for further exploration of novel compounds in the treatment of AD.

Oxidative stress and neuroinflammation are closely linked, forming a self-perpetuating cycle that exacerbates AD pathology. Oxidative stress, particularly in the presence of hydrogen peroxide, activates the NF-kB pathway, leading to the expression of proinflammatory cytokines and chemokines that activate leukocytes and provoke an immune response. The leukocytes then enter the tissue and produce ROS, further increasing oxidative stress. This cycle between oxidative stress and neuroinflammation exacerbates AD pathology and progression [28].

BV2 cells are a cell line of hippocampal microglial cells derived from C57BL/6 mice and immortalized with a v-raf/v-myc carrying J2 retrovirus. They are broadly used as a model for studying microglia in neurodegenerative diseases in vitro. Lipopolysaccharide, or LPS, is a glycolipid found in the cell wall of gram negative bacteria that is commonly used as a potent activator of immune cells, like microglia, in cell culture studies [29, 30]. LPS bind to toll-like receptor 4 (TLR4) on microglia to trigger the release of proinflammatory cytokines and ROS and morphological changes [31]. LPS-stimulated BV2 cells are commonly used to assess the potential of novel compounds as AD treatments. One study investigated the inhibitory potential of isorhamnetin, a flavonoid, against inflammation in LPS-Stimulated BV-2 cells. The results of the study indicated that isorhamnetin reduced production of proinflammatory mediators and cytokines, TNF-α and IL-1β, by disrupting the NF-κB signaling pathway. Additionally, isorhamnetin reduced LPS-induced ROS generation and TLR4 expression. The anti-inflammatory and antioxidant capacity of isorhamnetin suggests its potential as a therapeutic agent for AD [32]. The ability to utilize LPS-stimulated BV2 cells as a model for the activated

microglia characteristic of AD creates an opportunity to study to proinflammatory cytokines and inflammation on disease progression.

To model neuronal cells, HT22 cells may be used. HT22 cells are a cell line of immortalized mouse hippocampal neurons [33]. These cells may be used in cell culture to study AD pathology. To model cytotoxicity and oxidative stress, HT22 cells may be treated with glutamate, which is a neurotransmitter used by cells to communicate with one another. However, in the presence of too much glutamate, it causes cells to undergo oxidative stress and cell death [33]. As a result, this model system may be used to study the oxidative stress and neurodegeneration that occurs in AD.

As discussed, microglia in proinflammatory conditions induce processes characteristic of AD pathology, like oxidative stress, inflammation, and neurodegeneration in neurons by releasing pro-inflammatory cytokines and ROS. The connection between oxidative stress and neuroinflammation is a key component of AD pathology. This study is particularly interested in creating a culture system for studying AD that fills the gap in knowledge in how microglial cells and neuronal cells interact in disease pathology. We hypothesized that by treating HT22 cells with LPS-BV2 conditioned media, which likely would contain proinflammatory cytokines, the cytotoxicity of glutamate on HT22 cells would be exacerbated.

METHODS

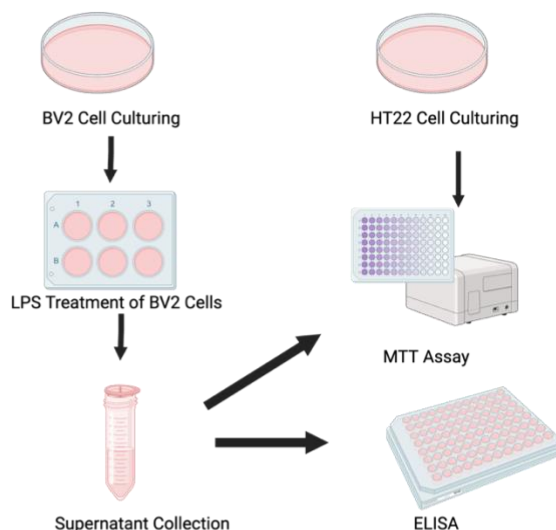


Figure 1: Experiment Schemata

Cell Culturing

BV2 Microglial Cells

BV2 microglial cells were grown on 10 cm tissue culture dished in complete cell medium containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM; Caisson Laboratories, Smithfield, UT), 5% Pen/Strep and 5% Fetal Bovine Serum (FBS). The cells were cultured in an incubator at 37ºC and 5% CO_2. Cells were monitored and passaged upon reaching 80% confluency. The medium was aspirated off the dish, and 3mL of complete media was added. The cells were scraped from the bottom of the dish using a scraper, and 1mL of the resuspended cells were then transferred to a new 10 cm dish with 9 ml of complete media. The dish was placed back in the incubator.

HT22 Neuronal Cells

HT22 mouse hippocampal neurons were also grown on 10 cm tissue culture dishes, and complete cell medium with 10 mL DMEM, 5% Pen/Strep, and 10% FBS was used. Upon reaching 80% confluency the cells were passaged. The media was aspirated off the dish, and the cells were washed with 5 mL PBS. Next, 3 mL trypsin was added to each plate, which was then placed in the incubator for 5 minutes. The cells and trypsin were collected into a 15 mL conical tube, which was then centrifuged for 10 minutes at 1.2 RCF to spin the cells into a pellet. The trypsin was aspirated off the cells, and the cells were resuspended in 3 mL of media. 1 mL of the resuspended cells was added to a 10 cm tissue culture dish containing 10 mL media. The dish was then placed back into the incubator.

LPS Treatment of BV2 Cells

To prepare for experiments, the BV2 cells were seeded in 6-well plates at 200,000 cells per well. The cell passage procedure was followed until the cells were lifted. Cells were pipetted into a 15 mL conical tube and centrifuged for 10 minutes at 1.2 RCF to spins the cells into a pellet. The supernatant was aspirated, and the pellet was resuspended in 2 mL of new media. 10 µL of the resuspended cells and 10 µL methylene blue were combined, and 10 µL of this solution was added to a hemocytometer to count the cells. The determined concentration was used to calculate the volume of the resuspended cells necessary to plate 200,000 cells in each well. Cells were seeded approximately 12 hours before treatment.

The lipopolysaccharide (LPS) used is from the Escherichia coli serotype 055:B5 (SigmaAldrich, St. Louis, MO). LPS was stored in the -20 ºC freezer and thawed in a 37º C bead bath. The LPS was diluted with complete media to the necessary concentrations. At treatment

time, the cells were treated with 0.05 µM of LPS for either four or eight hours. In another treatment group, cells were treated with LPS at 0.5 µM for 24 hours, after which the supernatant was collected. Another treatment group received a treatment of 0.5 µM LPS for 24 hours, after which they received an additional 24 hour 0.5 µM LPS treatment, totaling 48-hour treatment. A third group followed the same treatment, with an additional 24-hour LPS treatment, totaling 72 hours of treatment. After the treatment time was completed for each group, the supernatants were collected to determine the cytokines produced in the resulting inflammatory response. The supernatants were aliquoted into microfuge tubes, snap frozen on dry ice, and stored in the -20 ºC freezer.

Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

To measure the production of pro-inflammatory cytokines, an ELISA was performed on the supernatants for determine TNF-α levels. The BV2 supernatants were thawed and the assay was run using BioLegend ELISA MAX^{TM} Deluxe Set Mouse TNF- α (BioLegend, San Diego, CA). The ELISA was run in accordance with the instructions from the kit manufacturer and the assay was read using a BMG CLARIOstar Plus microplate reader (BMG Labtech, Cary, NC.) using ELISA kit settings.

MTT Assay for cell survival

Reagent Preparation

MTT was dissolved in Dulbecco's PBS at 5 mg/mL and filtered through a 0.22 µm bottle-top filter. MTT was stored in a foil-covered, sterile 50 mL conical tube at 4 degrees. Glutamate stock was prepared at 200 mM in H_2O, with addition of 5N NaOH to maintain a pH of 7, and the

stock was then diluted to desired concentrations using 5% FBS complete media. The supernatants collected from the LPS-BV2 treated cells were thawed in a 37ºC bead bath. For the groups treated with glutamate and supernatant, the mixture was prepared by diluting 200 mM glutamate with the collected supernatant, rather than media, to reach the desired glutamate concentrations.

Treatment	Components
40 mM Glutamate	30 µL 5% FBS Complete Media + 20 µL 200 mM Glutamate
20 mM Glutamate	40 µL 5% FBS Complete Media + 10 µL 200 mM Glutamate
40 mM Glutamate + Supernatant	30 µL Supernatant + 20 µL 200 mM Glutamate
20 mM Glutamate + Supernatant	40 µL Supernatant + 10 µL 200 mM Glutamate

Table 1: Description of components for each treatment of the MTT assay. The concentration of glutamate listed in the treatment column refers to the total concentration glutamate once 50 µL of the treatment is added to each well, which already contains 50 µL media

MTT Procedure

Cells were seeded in a 96-well dish at 5,000 cells per well, with 50 µL of media and maintained in the incubator. 24 hours after seeding, the treatments were added to the cells. The treatments were 40 mM glutamate, 20mM glutamate, supernatants only, 40 mM glutamate diluted in supernatant, and 20 mM glutamate diluted in supernatant. We used the previously mentioned supernatants that were collected from the LPS treated BV2 cells. 50 µL of the treatments were added to their respective wells in triplicates, and the plate was returned to the

incubator. Alternatively, the “no glutamate/ supernatant” group was treated with 50 μL media. 20 hours after the treatments were added, 10 μL of MTT was added to each well, and 10 μL media was added to the “No MTT” group. 4 hours later (24 hours after the treatments began) the MTT and media was removed from the wells and 100 μL of DMSO was added to each well. The plate was covered with tin foil and placed in an orbital shaker preheated to 37 ºC for 15 minutes at 500 rpm. The absorbance was then read at 570 nm by a FLUOstar Omega microplate reader (BMG Labtech). The positive control was the “no glutamate/ supernatant” group, and the negative control was the “no cells” group.” The blank was set to the “no MTT” group.

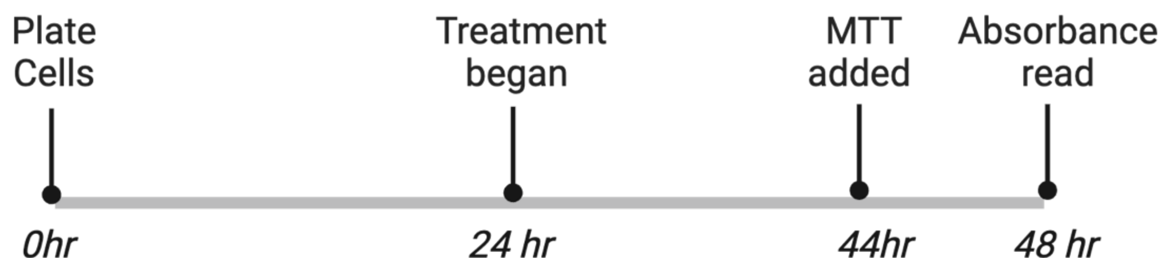


Figure 2: Timeline of MTT assay

RESULTS

Increasing levels of TNF-α following LPS treatment of BV2 cells

To confirm the production of proinflammatory cytokines, and thus the activation of BV2 cells and inflammation, BV2 cells were treated with 0, 0.0005, 0.005, 0.5, and 5 ug/mL LPS for either 4 or 8 hours, and TNF-α concentration in the supernatant was measured using an ELISA.

When treated with 0, 0.0005, 0.005, 0.5, and 5 ug/mL LPS (Figure 3), there was a general increase in TNF-α protein level in the BV2 supernatant. Additionally, 4-hour and 8-hour treatment times had no substantial impact on the concentration of TNF-α produced by the BV2 cells when the LPS concentration in the treatment is held constant. Replicates of these experiments have not been completed, so no statistical analysis can be conducted at this time.

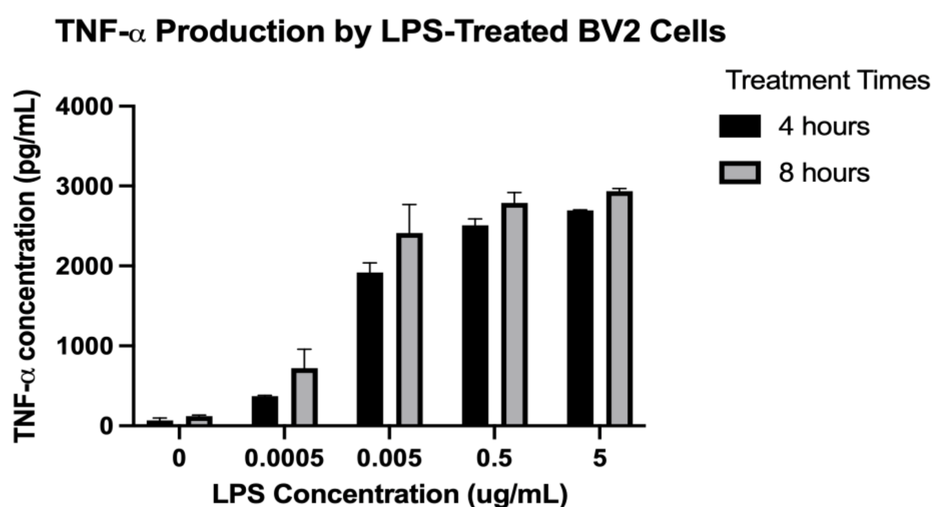


Figure 3. Peripheral cytokine production (pg/mL) in serum of BV2 cells. BV2 cells were treated with increasing concentrations of LPS for either 4 or 8 hours. Supernatants were collected, and TNF-α was measured using an ELISA. Error bars represent standard deviation of the mean.

Glutamate induces cytotoxicity in HT22 cells

To expand on previous findings about the cytotoxicity of glutamate on HT22 cells, we treated HT22 cells with 0, 1, 5, and 10 mM glutamate for 24 hours and measured cell survival (figure 4). Consistent

with published results, we found that increasing glutamate concentration in the treatment led to a decrease in cell viability [33].

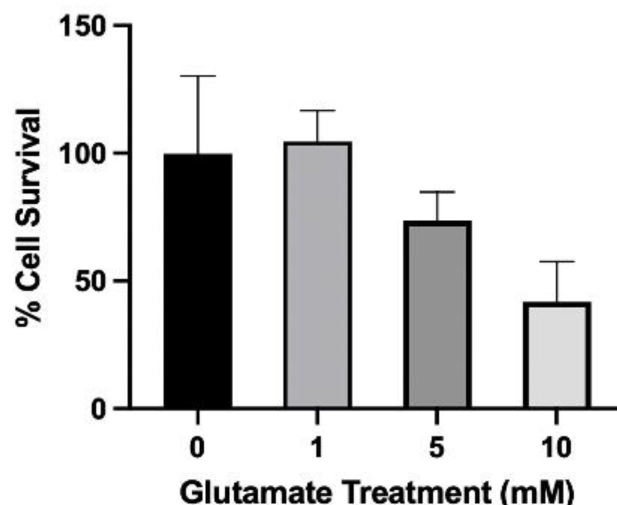
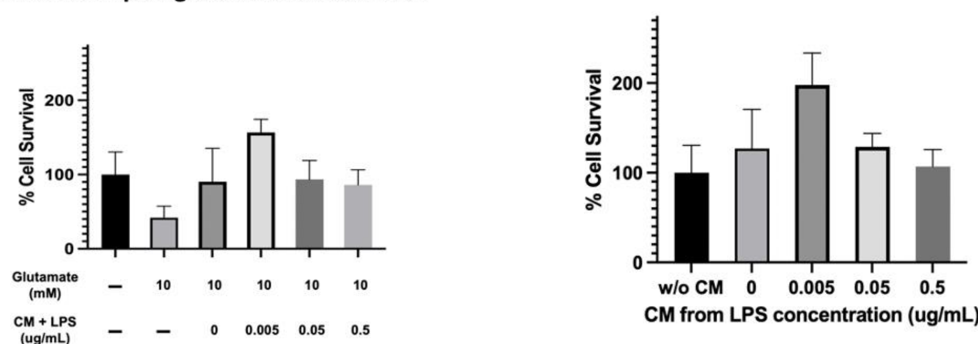


Figure 4. Cell survival following 24-hour Glutamate treatment. HT22 cells were treated with increasing concentrations of glutamate, and cell viability was measured using an MTT assay. Error bars represent standard deviation of the mean.

Cell viability following HT22 cell treatment with glutamate and LPS-treated BV2 conditioned media (CM).

To observe the combined effect of L PS-treated BV2 conditioned media and glutamate treatment on HT22 cell survival, HT22 cells were treated with 10 mM glutamate and conditioned media of BV2 cells treated with 0, 0.005, 0.05, and 0.5 uL/mL LPS and cell viability was measured using an MTT assay (figure 5). The 10 mM glutamate treatment was chosen as it induces cytotoxicity in about 50% of the HT22 cells following 24 hours of treatment (figure 4). Our results found that cotreatment of HT22 cells with glutamate and LPS-treated BV2 conditioned media led to a general increase in cell viability, especially with the group treated with the 0.005 ug/mL LPS treated BV2 conditioned media (figure 5a). To investigate whether the

increase in cell survival was due to BV2 CM being neuroprotective or instead enhancing cell proliferation, HT22 cells were treated with BV2 CM alone (figure 5b). Our results found that BV2 CM led to a general increase in cell survival compared to cells not treated with BV2 CM, particularly with the 0.005 ug/mL LPS-treated BV2 CM.



(a) **(b)**

Figure 5. Cell survival following (a) a 24-hour glutamate and BV2 conditioned media cotreatment and (b) a 24-hour BV2 conditioned media treatment. HT22 cells were treated with 10mM glutamate and/or CM collected from BV2 cells that were treated with various concentrations of LPS. Cell viability was measured in an MTT assay. Error bars represent standard deviation of the mean.

DISCUSSION

When BV2 cells were treated with 0, 0.0005, 0.005, 0.5, and 5 ug/mL LPS for 4 and 8 hours, we saw a general increase in TNF-α production as LPS concentration in the treatment increased. We also found that there was little change in TNF-α production when LPS treatment time increased from 4 hours to 8 hours. The increase in TNF-α production in response to LPS indicates that the LPS is effectively stimulating the BV2 cells and causing inflammation to occur. These results are consistent with previous research that indicates that stimulating BV2 cells with LPS leads to the production of pro-inflammatory cytokines, as it has been shown that the LPS

[34]stimulation leads to IL-6 and TNF-α production previously . The confirmed inflammation of the BV2 cells ensures that the supernatants used for treatment of the HT22 cells in our MTT assays contain proinflammatory cytokines, as well as other products that BV2 cells produce when activated. This is inflammation in part is thought to mimic what occurs in AD, supporting our hypothesis that we would observe enhanced cytotoxicity of HT22 cells when cotreated with BV2 CM and glutamate.

When HT22 cells were treated for 24 hours with 0, 1, 5, and 10 mM glutamate alone, there was a decrease in cell viability as the concentration of glutamate increased. This result is what was expected, and it is consistent with previous literature as glutamate is known to reduce cell viability by inducing oxidative stress [33]. Because the results are consistent with previous research, the function of our MTT assay and the quality of glutamate were confirmed. Because the 10 mM glutamate treatment produced approximately 50% decrease in cell viability, we decided to use a 10 mM glutamate treatment for our co-treatments for with BV2 conditioned media.

When HT22 cells were cotreated with BV2 conditioned media, the cytotoxic effect of glutamate was ameliorated compared to HT22 cells treated with glutamate alone. All cotreated groups saw this trend, including the cells cotreated with conditioned media from BV cells that did not receive LPS. The biggest increase in cell viability occurred in the group that was cotreated with supernatant of the BV2 cells that were treated with 0.005 ug/mL LPS. These results contradicted our hypothesis, as we expected that the supernatant would exacerbate the cytotoxicity caused by glutamate due to the presence of proinflammatory cytokines. The proinflammatory cytokines produced by microglial cells contribute to neuroinflammation, an important component of the AD pathology. Because glutamate causes oxidative stress in HT22

cells, the cotreatment of BV2 conditioned media and glutamate were expected to model AD pathology and cause increased cytotoxicity in HT22 cells. This means that our hypothesis, that LPS treated BV2 CM would exacerbate cell death in HT22 cells caused by glutamate treatment, was not supported by our results. These results raise a number of questions, one of which is, does the BV2 conditioned media lead to increased cell viability because it is neuroprotective or because it is enhancing cell proliferation? In other words, is the BV2 conditioned media preventing glutamate from causing cell death, or is it causing the HT22 cells to multiply more rapidly?

To investigate these questions, we treated HT22 cells with BV2 conditioned media alone and measured cell viability. Our results showed that treating HT22 cells with BV2 conditioned media may maintain the number of viable cells, or, in some cases, increase the number of viable cells. Because the cell viability may be greater even when there was nothing for the supernatant to "protect" the HT22 cells from, supports the idea that the BV2 supernatants increase cell viability by enhancing cell proliferation rather than by being neuroprotective.

Future Directions

In both the groups treated with the BV2 conditioned media alone and the BV2 conditioned media and glutamate, the groups that may have the highest cell viability were the groups treated or cotreated with 0.005 ug/mL LPS-treated BV2 conditioned media. This apparent finding is interesting because we demonstrated roughly equal concentrations of proinflammatory TNF-α in cells treated with 0.005 ug/ml LPS and the BV2 CM treated with higher LPS concentrations. With these results in mind, future research should determine what component of the BV2 CM led to an increase in cell viability. Potential culprits would be microglial secreted growth factors, like brain derived neurotropic factor and nerve growth factor, among other

known microglial products. These factors have all been shown to be secreted by BV2 cells when stimulated with LPS, and they also have been shown to enhance HT22 cell viability [35-37]. The levels of these proteins could be determined in the supernatant using a western blot, or gene expression of these proteins could be determined by running and reverse transcription polymerase chain reaction (RT-PCR) of the BV2 cell lysates. Furthermore, to confirm that the causative agent is a protein, the supernatant could be heated to denature the proteins. This supernatant could then be used to treat the HT22 cells. If the cell viability enhancement effect remains, then the causative agent is likely not a protein or microglial secreted growth factor.

A coculture of BV2 cells and HT22 cells may also be performed to determine if the proximity of BV2 cells and HT22 cells affects the outcome of BV2 CM on HT22 cell viability. This model may also more closely mirror AD pathology, as microglial cells and HT22 cells are in close proximity in the brain.

CONCLUSION

The goal of this experiment was to create a culture system that fills the gap in knowledge regarding how microglial cells and neuronal cells interact in AD pathology and would thus provide us a model system to test potential therapeutic compounds. We hypothesized that due to the presence of proinflammatory cytokines in LPS-treated BV2 supernatants, and the role of proinflammatory cytokines in neuroinflammation and AD, the LPS-treated BV2 supernatants would exacerbate glutamate-induced cytotoxicity in HT22 cells. We found that despite the presence of proinflammatory cytokines in the BV2 supernatants, the supernatants may enhance cell viability of glutamate-treated HT22 cells possibly by enhancing cell proliferation. With these

findings in mind, future research should investigate what component of the BV2 CM enhances cell proliferation and if proximity of BV2 cells and HT22 cells affects cell viability.

REFERENCES

1. *Alzheimer's disease facts and figures*.
2. Butterfield, D.A. and C.M. Lauderback, *Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress.* Free Radic Biol Med, 2002. **32**(11): p. 1050-60.
3. *What Happens to the Brain in Alzheimer's Disease?* 2024.
4. Sehar, U., et al., *Amyloid Beta in Aging and Alzheimer's Disease.* International Journal of Molecular Sciences, 2022. **23**(21): p. 12924.
5. Wärmländer, S.K.T.S., et al., *Metal binding to the amyloid-β peptides in the presence of biomembranes: potential mechanisms of cell toxicity.* JBIC Journal of Biological Inorganic Chemistry, 2019. **24**(8): p. 1189-1196.
6. Weintraub, M.K., et al., *Imatinib methanesulfonate reduces hippocampal amyloid-beta and restores cognitive function following repeated endotoxin exposure.* Brain, Behavior, and Immunity, 2013. **33**: p. 24-28.
7. Gardner, L.E., et al., *Imatinib methanesulfonate reduces hyperphosphorylation of tau following repeated peripheral exposure to lipopolysaccharide.* Neuroscience, 2016. **331**: p. 72-7.

8. Gong, C.X. and K. Iqbal, *Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease.* Curr Med Chem, 2008. **15**(23): p. 2321-8.

9. Medeiros, R., D. Baglietto-Vargas, and F.M. LaFerla, *The role of tau in Alzheimer's disease and related disorders.* CNS Neurosci Ther, 2011. **17**(5): p. 514-24.

10. Forman, H.J. and H. Zhang, *Targeting oxidative stress in disease: promise and limitations of antioxidant therapy.* Nature Reviews Drug Discovery, 2021. **20**(8): p. 652-652.

11. Juan, C.A., et al., *The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies.* Int J Mol Sci, 2021. **22**(9).

12. *Oxidative Stress: what is, causes, effects and reduction*. 2024 2024]; Available from: https://corconinternational.com/oxidative-stress-what-is-causes/.

13. Ayala, A., M.F. Muñoz, and S. Argüelles, *Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal.* Oxidative Medicine and Cellular Longevity, 2014. **2014**(1): p. 360438.

14. Su, L.-J., et al., *Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis.* Oxidative Medicine and Cellular Longevity, 2019. **2019**(1): p. 5080843.

15. Grintzalis, K., et al., *Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides.* Free Radical Biology and Medicine, 2013. **59**: p. 27-35.

16. Boyd, S.D., et al., *Copper Sources for Sod1 Activation.* Antioxidants (Basel), 2020. **9**(6).

17. Reichert, C.O., et al., *Ferroptosis Mechanisms Involved in Neurodegenerative Diseases.* International Journal of Molecular Sciences, 2020. **21**(22): p. 8765.

18. Leng, F. and P. Edison, *Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here?* Nature Reviews Neurology, 2021. **17**(3): p. 157-172.

19. Zhou, S., et al., *An insight into the TAM system in Alzheimer's disease.* International Immunopharmacology, 2023. **116**: p. 109791.

20. Hansen, D.V., J.E. Hanson, and M. Sheng, *Microglia in Alzheimer's disease.* J Cell Biol, 2018. **217**(2): p. 459-472.

21. Doens, D. and P.L. Fernández, *Microglia receptors and their implications in the response to amyloid β for Alzheimer's disease pathogenesis.* Journal of Neuroinflammation, 2014. **11**(1): p. 48.

22. Steen, B.M., L.C. Gerstenfeld, and T.A. Einhorn, *Chapter 17 - The Role of the Immune System in Fracture Healing*, in *Osteoimmunology (Second Edition)*, J. Lorenzo, et al., Editors. 2016, Academic Press: San Diego. p. 297-310.

23. Fruhwürth, S., H. Zetterberg, and S.R. Paludan, *Microglia and amyloid plaque formation in Alzheimer's disease- Evidence, possible mechanisms, and future challenges.* Journal of Neuroimmunology, 2024. **390**.

24. Miao, J., et al., *Microglia in Alzheimer's disease: pathogenesis, mechanisms, and therapeutic potentials.* Front Aging Neurosci, 2023. **15**: p. 1201982.

25. Rodríguez, J.J., et al., *Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease.* Cell Death & Disease, 2010. **1**(1): p. e1-e1.

26. Ahmed, S., et al., *Taurine reduces microglia activation in the brain of aged senescence-accelerated mice by increasing the level of TREM2.* Scientific Reports, 2024. **14**(1): p. 7427.

27. Martín-Moreno, A.M., et al., *Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease.* Mol Pharmacol, 2011. **79**(6): p. 964-73.

28. Vaziri, N.D. and B. Rodríguez-Iturbe, *Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension.* Nat Clin Pract Nephrol, 2006. **2**(10): p. 582-93.

29. Li, C., et al., *Characterization of Inflammatory Signals in BV-2 Microglia in Response to Wnt3a.* Biomedicines, 2023. **11**(4).

30. Pellissier, H., *Use of O-glycosylation in total synthesis.* Tetrahedron, 2005. **61**(12): p. 2947-2993.

31. Sarkar, S., et al., *Characterization and comparative analysis of a new mouse microglial cell model for studying neuroinflammatory mechanisms during neurotoxic insults.* NeuroToxicology, 2018. **67**: p. 129-140.

32. Kim, S.Y., et al., *Isorhamnetin alleviates lipopolysaccharide-induced inflammatory responses in BV2 microglia by inactivating NF-κB, blocking the TLR4 pathway and reducing ROS generation.* Int J Mol Med, 2019. **43**(2): p. 682-692.

33. Shen, G.N., et al., *Knockdown of peroxiredoxin V increases glutamate-induced apoptosis in HT22 hippocampal neuron cells.* Molecular Medicine Reports, 2018. **17**.

34. Zhu, J., et al., *COX-2 contributes to LPS-induced Stat3 activation and IL-6 production in microglial cells.* Am J Transl Res, 2018. **10**(3): p. 966-974.

35. Nakajima, K., et al., *Neurotrophin secretion from cultured microglia.* J Neurosci Res, 2001. **65**(4): p. 322-31.

36. Pansri, P., et al., *Brain-derived neurotrophic factor increases cell number of neural progenitor cells derived from human induced pluripotent stem cells.* PeerJ, 2021. **9**: p. e11388.

37. Farrell, E., et al., *Inducing neuronal characteristics of HT22 cells through differentiation with pro-neural factors.* Physiology, 2023. **38**(S1): p. 5732921.