Enhanced Mitochondrial Reductive Stress and Cell Death Observed Via the Synergistic Effect of Glucose Starvation and Ceftriaxone/N-acetylcysteine Treatment on Human Glioma Cells

Research Plan

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A. ***Rationale:***

Gliomas derived from glial cells within the human central nervous system are recognized as being among some of the deadliest human cancers. To date, the only treatment that exists for these tumors are surgery, radiation therapy, and chemotherapy. Despite treatment, patients with glioma tend to have poor prognosis, and median survival time after diagnosis is generally within 1.5 to 3 years. Previous studies have suggested the importance of antioxidants in cancers as an effect of the dysregulated metabolism of cancer cells. The metabolic reprogramming that occurs with oncogenic onset generally leads to increased levels of oxidative stress, thus forcing cancer cells to rely on increased antioxidant concentration in order to alleviate this stress; glioma cells are no different. It has long been hypothesized that this vulnerability could be exploited to terminate cancerous cells. Past studies have suggested that increases in intracellular antioxidant concentration are correlated with increased mitochondrial reductive stress and cytotoxicity. Mitochondrial reductive stress- induced cytotoxicity is characterized by paradoxical oxidative stress induced by the disparity between increased intracellular antioxidant concentrations and decreased reactive oxygen species concentration within the cell. Glutathione (GSH) has shown to be an important factor in tumorous and non tumorous cells alike due to its antioxidant properties. GSH is composed of three different substances: cysteine, glutamate and glycine. Intracellular GSH concentrations may be increased by increasing intracellular concentrations of both cysteine and glutamate. Past research has shown how *N*-Acetylcysteine (NAC) is a precursor of cysteine. Additionally, intracellular glutamate concentration may be increased through upregulation of one of its key transport proteins: Glutamate transporter protein 1 (GLT-1). Previous research has shown how beta lactam antibiotics, such as ceftriaxone (CTX), lead to the upregulation of GLT-1, which therefore increases intracellular glutamate concentrations. However, whether or not these two substances, CTX and NAC, are able to work synergistically in order to elevate mitochondrial reductive stress and ultimately cell death remains unknown.

B. Research Questions/Hypothesis/Goals/ Expected Outcomes:

Research Questions:

Problems that I hope to address with my research include:

1. Will glucose starvation and CTX/NAC administration lead to mitochondrial reductive stress?
2. Will glycolysis increase as a result of this stress?

Hypothesis:

Prior studies have indicated how increased mitochondrial reductive stress may lead to cell death. Although previous research has not utilized CTX and NAC in conjunction to cause mitochondrial reductive stress and cell death, using these two substances for a synergistic effect with glucose starvation of cells may lead to the mitochondrial reductive stress that has been shown to cause cell death. Therefore, this research will be initiated with the hypothesis that an increase in intracellular glutathione concentration due to CTX/NAC administration in conjunction with glucose deprivation will lead to mitochondrial reductive stress and cell death, and thus a new route for glioma treatment and novel therapeutic development.

Goals:

My research aims to create a pathway for new research towards cancer treatment, as well as a foundation for drug development utilizing mitochondrial reductive stress as a means of terminating cancer

cells. Additionally, I aim to confirm my hypothesis by viewing decreases in mitochondrial function as well as increases in glycolysis.

Expected Outcomes:

I expect my research to show decreases in mitochondrial respiration as well as increases in glycolysis through changes in the extracellular environment. I also expect CTX/NAC to work more effectively with glucose deprivation than without.

C. *Procedure, Risk and Safety, Data Analysis:*

I. Procedure:

Western Blotting:

$1x10^6$ 667 glioma cells will be harvested from 667 cell media (for 200mL: 200mL TSM base, 4mL 27-A, 200µL heparin, 160µL EGF, 160µL FGF) by centrifugation (1000 RCF, 3 minutes) after treatment with CTX/NAC (1mM CTX, 10mM NAC) for set time intervals (30 minutes, 1 hour, 8 hours, and 24 hours). Cell lysates will be treated with 70µL PBS to disrupt cellular membrane and release cell contents. Permeabilized cells will be centrifuged for 15 minutes with 16000 RCF to separate cytosolic and membrane proteins. Supernatant with cytosolic proteins will be transferred to a new tube. Pellet will be solubilized using 50µL CST and resuspended using a pipette. Tubes will then be incubated at 4C for 30 minutes with constant mixing. Lysates will then be centrifuged at 16000 RCF for 15 minutes at 4C. Supernatant containing membrane proteins will be transferred to a new tube. 2µL of each lysate will be added to cuvettes containing 1mL of Bradford Reagent each. Standards will be made using BSA. Mixtures will then be measured spectrophotometrically in order to obtain protein concentration. Once protein concentrations have been obtained, sample buffers will be made for each lysate (4X NuPage LDS Sample Buffer, 1X CST/PI [10X CST, 100X PI], 10X DTT). Sample buffer-lysate mixture will be heated at 70C for 10 minutes before shorted in centrifuge until 8000 RCF. Samples will then be transferred to 24 well NuPage 10% polyacrylamide gel for separation by gel electrophoresis using running buffer (1L = 50mL 20X MOPS, 950 mL dH_2O). Samples will be run for 1.5 hours at 15V before gel is transferred to membrane using transfer buffer (1L = 3.03g Trizma Base, 14.41g glycine, 800mL dH_2O, 200mL methanol). Gel-membrane apparatus will be run at 220mA for 2 hours. After transfer, the membrane will be washed with TBST and blocked for 30 minutes in 10% skim milk. Primary antibody Glut1 will be added and allowed to incubate for 2 hrs. Antibody will be removed and membrane will be washed 3 times for 15 minutes each with TBST. Secondary rabbit antibody will be added and allowed to incubate for 1 hour at room temperature. Antibody will be removed and the membrane will be washed with TBST 3 times for 15 minutes each. Chemiluminescent substrates (SuperSignal™ West Femto Maximum Sensitivity Substrate) will be used to detect the protein followed by film exposure to visualize the specific protein transferred onto the membrane.

Lactate Assay:

667 glioma cells will be harvested from 667 cell media (for 200mL: 200mL TSM base, 4mL 27-A, 200µL heparin, 160µL EGF, 160µL FGF) before treatment with CTX/NAC (1mM CTX, 10mM NAC) for set time points (0-60 minutes, n=7). 96 well plate with a flat bottom will be used. 250µL buffer solution (.5M glycine, .4M hydrazine, pH will be adjusted to 9.0 with 4N KOH), 20µL supernatant fluid or 20µL water (blank) or 20µL uninoculated medium, 25µL NAD, 2.5µL L-LDH will be added to each well. Samples will be incubated at 25C for 1 hour inside Spectramax. Optical density will be measured at wavelength 340nm with Spectramax. Optical densities will then be used to quantify NADH concentration. Lactate concentration will be determined using NADH concentration values. Values will then be plotted in ImageJ with standard error bars.

Seahorse Assay:

In the day prior to the assay, cell seeding density will be determined to be between $5x10^3$ to $5x10^4$ 667 human glioma cells per well. Sensor cartridge will then be hydrated overnight using utility plate. Each probe of sensor cartridge will be submerged in 200µL sterile tissue culture grade water and left to hydrate in a non-CO_2 chamber at 37C overnight. In the day of the assay, sensor cartridge will be removed from the incubator and water from utility plate will be disposed of. Wells of utility plate will then be filled with 200µL XF Calibrant (included in Seahorse XF Analyzer Kit) before submerging each probe of sensor cartridge in utility plate. Sensor cartridge and utility plate will then be placed in a non-CO_2 37C incubator for 45-60 minutes prior to loading injection ports of sensor cartridge. XF Assay medium will then be prepared (XF DMEM Medium pH 7.4, XF Pyruvate [100mM], XF L-Glutamine [200mM]) and incubated at 37C. Cells will be harvested from 667 cell media (for 200mL: 200mL TSM base, 4mL 27-A, 200µL heparin, 160µL EGF, 160µL FGF) and transferred to a conical tube. Cells will be centrifuged at room temperature with 200 RCF for 5 minutes. While cells are centrifuged, 50µL XF Assay medium will be added to control wells of room temperature cell-tak-coated Seahorse XF96 cell culture plate. Supernatant will be aspirated from conical tube and cells will be resuspended in assay medium to desired concentration of cells per well in 50µL assay medium. 50µL of cell suspension will be pipetted into culture plate wells, excluding control wells. Plate will then be centrifuged at room temperature with 200 RCF and 0 braking before plate is incubated in non-CO_2 chamber. 130µL assay medium will be added to each well of culture plate. Cell plate will then be placed in non-CO_2 37C incubator for 15-25 minutes. 20µL CTX, NAC, CTX/NAC (CTX: 1mM, NAC: 100mM) and PBS will be added to specified injection ports of sensor cartridge. Sensor cartridge and cell culture plate will be inserted into Seahorse XFe96 Analyzer and run for its duration (~6 hours). Results will be analyzed and imaged using ImageJ software.

II. Risk and Safety:

1) **Human subjects:** N/A

2) **Vertebrate animals:** N/A

3) **Potentially hazardous biological agents (PHBA):**
The 667 Human Glioma cell line that had been previously gifted to the Cantley Laboratory at Weill Cornell Medicine from the Memorial Sloan Kettering Cancer Center will be utilized and handled in a biological safety cabinet under proper supervision. Appropriate safety equipment will be used, including gloves, goggles, lab coats, long pants and closed toed shoes. This requires biosafety level 2 practices and containment facilities. Tissue culture wastes including tissue media will be treated with bleach (a final concentration of >10%) for 30 minutes and discarded into the laboratory sink. The sink will be thoroughly rinsed after waste discharge.

4) **Hazardous chemicals/activities/devices:**
Safety precautions must be taken when handling chemicals, and appropriate protective attire will be used at all times. These include gloves, goggles, lab coats, closed toed shoes, and long pants. Hazardous wastes will be properly disposed of in designated containers and will be routinely collected by the Weill Cornell Medicine Department of Environmental Health and Safety. Reagents will be handled with care as described below:

Glycine:

It may cause irritation to the eyes and skin through contact. If inhaled, may cause irritation to the respiratory tract. If ingested, glycine may cause irritation to the digestive tract. If one's eye comes in contact with glycine, one can use water to flush out the chemical, washing for at least 15 minutes. In the

case of skin irritation, one can use water to wash off the chemical, washing for at least 15 minutes. In the case of ingestion, one should seek medical attention. In the case of inhalation, receive artificial respiration if necessary and move to fresh air.

Hydrazine Sulphate:
It may cause irritation to the eye and skin through contact, respiration tract irritation through inhalation, allergic skin reactions and digestive tract irritation if swallowed. If inhaled, one should move to fresh air and be kept at rest. If in contact with skin, one should wash with soap and water for at least 15 minutes. If swallowed, mouth should be rinsed with water. In all cases, seek medical attention and poison control should irritation persist.

Potassium Hydroxide:
It is incredibly toxic and will cause severe burns to the skin and eye when in contact, circulatory system failure and digestive tract perforation if swallowed and pulmonary edema if inhaled. In the case of eye contact, eye(s) should be flushed with fresh water for at least 15 minutes. In the case of skin contact, contaminated clothing should be removed and skin should be washed with water for at least 15 minutes. If ingested and alert, 2-4 cups of water or milk should be ingested. Do not attempt if unconscious. Do NOT induce vomiting. If inhaled, move to fresh air immediately. In all cases, seek medical attention and poison control.

L-Lactate Dehydrogenase:
It may cause eye, skin and respiratory tract irritation. It may be harmful if inhaled, ingested, or absorbed by skin. In the case of eye contact, one should flush eye(s) with water for at least 15 minutes. In the case of skin contact, skin should be washed with water for at least 15 minutes. In the case of inhalation, one should be moved to an area of fresh air and given artificial respiration if necessary. In the case of ingestion, one should wash out mouth provided that one is conscious. In all cases, seek medical attention and poison control should irritation persist.

Nicotinamide Adenine Dinucleotide (NAD):
NAD may be irritating to the mucous membranes and upper respiratory tract. NAD may be harmful by inhalation, ingestion, or skin absorption, and may cause eye, skin, or respiratory system irritation. In the case of inhalation, move to fresh air. If not breathing, give artificial respiration or give oxygen by trained personnel. In the case of skin contact, immediately wash skin with soap and plenty of water for at least 15 minutes. Remove contaminated clothing. In the case of eye contact, hold eyelids apart and flush eyes with plenty of water for at least 15 minutes. Have eyes examined and tested by medical personnel. In the case of ingestion, wash out mouth with water provided one is conscious. In all cases, seek medical attention and poison control if irritation persists.

Dithiothreitol (DTT):
DTT will cause serious eye and skin irritation when in contact, and is harmful if swallowed. In the case of ingestion, mouth should be flushed with water for at least 15 minutes, and poison control should be called immediately. If in contact with skin, skin should be washed thoroughly with soap and water. If in contact with eyes, hold eyelids apart and flush eyes with plenty of water for at least 15 minutes. Have eyes examined and tested by medical personnel. In all cases, seek medical attention and poison control if irritation persists.

Phosphate Buffer Solutions (PBS):

It may cause irritation to the eyes and skin through contact. If ingested, PBS may cause irritation to the digestive tract. If inhaled, it may cause irritation to the respiratory tract. If one's eye comes in contact with PBS, one can use fresh water to flush out the chemical. In the case of skin irritation, one may wash skin with soap and water. In the case of ingestion, one may rinse mouth with water.

Bradford Reagent:
Bradford Reagent will cause severe skin burns and eye damage while in contact, may cause respiratory irritation if inhaled, is harmful if swallowed and may cause damage to internal organs. If inhaled, one should move to an area of fresh air. If in contact with skin, one should remove contaminated clothing and should thoroughly wash skin with fresh water for at least 15 minutes. If in eyes, one should rinse cautiously with water. If ingested, one should flush mouth with water. Do NOT induce vomiting. In all cases, seek medical attention or poison control should symptoms persist.

Bovine Serum Albumin (BSA):
BSA may be harmful if swallowed, and may cause irritation to the mucous membranes and upper respiratory tract if inhaled. BSA may also cause eye and skin irritation when in contact. In the case of inhalation, remove to fresh air. If not breathing, give artificial respiration or give oxygen by trained personnel. In the case of skin contact, immediately wash skin with soap and plenty of water for at least 15 minutes. Remove contaminated clothing. In the case of eye contact, hold eyelids apart and flush eyes with plenty of water for at least 15 minutes. Have eyes examined and tested by medical personnel. In the case of ingestion, wash out mouth with water provided one is conscious. Do NOT induce vomiting unless directed to do so by a medical professional.

NP LDS Sample Buffer (4X):
NP LDS Sample Buffer may cause irritation to the eyes and skin when in contact, and may cause respiratory tract irritation when inhaled. If inhaled, one should move to an area of fresh air. In the case of eye contact, one should flush eyes with fresh water for at least 15 minutes. In the case of skin contact, one should wash area with fresh water. If ingested, one should rinse mouth with water. Do NOT induce vomiting unless directed to do so by a medical professional.

Tris Buffered Saline with Tween (TBST):
It may cause eye, skin and respiratory tract irritation. It may cause irritation if inhaled, ingested, or absorbed by skin. In the case of eye contact, one should flush eye(s) with water for at least 15 minutes. IN the case of skin contact, skin should be washed with water for at least 15 minutes. in the case of inhalation, one should be moved to an area of fresh air and given artificial respiration if necessary. In the case of ingestion, one should wash out mouth provided that one is conscious. In all cases, seek medical attention and poison control.

Cell Signaling Technology Buffer (CST):
CST may cause irritation to the eyes and skin when in contact, and may cause irritation to the respiratory tract and digestive tract when inhaled or ingested. In the case of eye contact, one should flush eye with fresh water for at least 15 minutes. In the case of skin contact, one should wash hands thoroughly with soap and water. In the case of ingestion, one should wash mouth with fresh water. Do NOT induce vomiting. In the case of inhalation, one should move to an area of fresh air.

4-Morpholinepropanesulfonic acid (MOPS):
MOPS will cause skin irritation and serious eye irritation when in contact. MOPS may cause respiratory tract irritation when inhaled. If inhaled, one should move to an area of fresh air and be given artificial

respiration if necessary. If in contact with skin, one should wash the affected area with plenty of water. If in contact with eyes, one should rinse cautiously with water for several minutes. In all cases, if irritation persists one should seek medical attention and/or poison control.

Dulbecco's Modified Eagle Medium (DMEM):
DMEM is a common medium used for tissue culture cells. It may cause irritation to the eyes, skin, mucous membranes and respiratory tract if inhaled. If eye irritation occurs, one should wash thoroughly with water and seek medical attention. In the case of skin irritation, one should wash the area with soap and plenty of water. If swallowed, one should rinse mouth with water.

Protease Inhibitor (PI):
PI can cause both skin irritation and serious eye irritation. In the case of eye contact, one should flush eye with fresh water for at least 15 minutes. In the case of skin contact, one should wash hands thoroughly with soap and water. Should irritation persist, one should seek medical attention and/or poison control.

Methanol:
Methanol is a mild to moderate eye irritant. Inhalation, ingestion or skin absorption of methanol can cause significant disturbances in vision, including blindness. In the case of skin contact with methanol, one should wash the area immediately with soap and water for at least 15 minutes. In the case of eye contact with methanol, one should flush the affected eye with water immediately. In the case of ingestion, seek medical attention immediately. Do NOT induce vomiting unless instructed to do so by a medical professional.

Seahorse XF Analyzer Buffers:
These buffers are included in the Seahorse XF96 Extracellular Flux analyzer kit and may cause skin and eye irritation upon exposure. Proper protective gear including a lab coat, goggles, gloves and closed toed shoes should be worn at all times.

Ceftriaxone (CTX):
CTX is an antibiotic that may cause allergic reaction, asthma symptoms, or breathing difficulties when inhaled. CTX causes skin and eye irritation when in contact. In the case of eye contact, one should flush eye with fresh water for at least 15 minutes. In the case of skin contact, one should wash hands thoroughly with soap and water. In the case of allergic reaction or asthma symptoms, one should seek medical attention.

***N*-Acetylcysteine (NAC):**
It may cause irritation to the eyes and skin through contact. If ingested, NAC may cause irritation to the digestive tract. If inhaled, it may cause irritation to the respiratory tract. If one's eye comes in contact with NAC, one can use fresh water to flush out the chemical. In the case of skin irritation, one may wash skin with soap and water. In the case of ingestion, one may rinse mouth with water.

III. Data Analysis:

1) **Western Blotting:**
   Protein bands will be visualized using the west femto chemiluminescent method, and analyzed based on their molecular weights and band intensities. Bands will be imaged using Image Lab software.
2) **Lactate Assay:**

NADH concentrations will be determined spectrophotometrically at 340nm with Spectramax software. Concentrations will be quantified to determine L-lactate concentrations and will be imaged in ImageJ.

3) **Seahorse Assay:**
Seahorse assay will be analyzed via the Seahorse XF96 Analyzer. Results will be quantified using ImageJ.
4) **Quantification Analysis:**
The standard error of the mean will be calculated using Excel software and error bars will be shown in the results graph.

D. Bibliography:

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– No Appendix exist –