Using Antioxidants to Remediate Oxidative Stress, Motility, and ASH neuronal death in Huntington's model of *C.elegans.*

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Translational Medical Science

A. Rationale

According to the Huntington's disease Society of America (HDSA) in 2018, there are approximately 30,000 Americans with Huntington's disease (HD) symptoms and 200,000 Americans at risk of inheriting HD. HD is a neurodegenerative disease with no cure and is characterized by progressive deterioration of nerve cells in the brain. The loss of nerve cells leads to physical and mental disorders like involuntary movements and slurred/slowed speech (HDSA, 2018). There are established medicines like tetrabenazine, Haldol, Celexa, and Prozac which are prescribed to HD patients to lessen the physical and mental effects of the disease. However, these medicines are highly ineffective because they have severe side effects like low blood pressure, dystonia, and mood swings along with being highly addictive. HD patients will typically die 10 to 30 years after diagnosis from pneumonia, HD related injuries, and/or heart complications (Mayo Clinic 2018).

HD can be characterized into two major categories which HD and juvenile HD. Both are very similar, but are different based off of time when patients are diagnosed with the disease. HD patients will typically experience difficulty focusing, insomnia, abnormal eye movements, muscle rigidness (dystonia), and impaired speech. Juvenile HD patients will typically experience seizures, tremors, minute involuntary movements, and a decrease in grades and performance in school. Even though both varieties of HD are for two different life stages, both types of patients experience physical, cognitive, and mental disorders. HD is a genetic disease because the HD gene can be inherited onto the carriers' offspring. HD is an autosomal dominant disorder which basically means that an individual only needs one defective gene from their parents in order to develop HD. For example, if one parent has the HD gene and one parent does not the offspring still has a chance of inheriting the disease. (Mayo Clinic, 2018)

HD is a neurodegenerative disease that results from the repetition cytosine-adenine-guanine (CAG) protein which creates longer polyQ tracks which exacerbates the disease (HC et al, 2014). HD is the result of the repetition of the cytosine-adenine-guanine (CAG). In a healthy human the CAG protein is typically repeated 10 to 35 times within a gene. However, HD patients will have CAG repetition 36 to 120 times. The abnormally long CAG

segment will lead to the production of the Huntington protein and this long protein is the Htt mutation. This protein will then be cut into smaller fragments that will bind together and affect the normal function of the neurons in the cells. The neurons will then start to die causing the brain to exhibit and cause the signs and symptoms of HD. When the rate of neuronal death increases, there is also an increase in the rate of oxidative stress. (HDSA, 2018).

Oxidative stress is a condition when the body is going through an extreme level of reactive oxygen species (ROS) production. A free radical is a type of molecule that has one or more unpaired electrons. ROS is an example of a free radical. The ROS production exceeds the counteracting antioxidants which causes an imbalance in the body. This imbalance damages lipids, proteins, and DNA. An extreme rate of oxidative stress leads to an increase in the rate of ASH neuronal death which worsens the mutation. Antioxidants have the ability to donate an electron to free radicals as an attempt to restore equilibrium to the cells. Antioxidants have neuroprotective and anti-oxidation properties which is why they are so beneficial to the human body. (Cao et al, 2007)

Oxidative stress can be relieved by use of anti-oxidants. Gingko biloba, EGCG, and Salidroside are all antioxidants that possess neuroprotective and anti-oxidation properties, Ginkgo biloba is native plant to Asian countries like China. The leaves from the Gingko tree are known for having beneficial medicinal properties after ingestion. Gingko also contains high levels of antioxidants in the leaves. When Gingko Biloba is applied to the cells undergoing oxidative stress a temporary balance could be restored. Lastly, Gingko promotes better blood circulation which could protect the brain from any cognitive and neural damage which is often compromised in HD patients. (Cao et al, 2007)

Epigallocatechin Gallate (EGCG) is a natural forming compound commonly found in green tea. EGCG is the most abundant catechin, which is a type of antioxidant, in green tea. EGCG has been used in the past to act as a potential solution in neurodegenerative and cardiovascular diseases along with having anti-carcinogenic and anti-inflammatory properties. EGCG is plentiful with antioxidants similar to Ginkgo. Again by applying EGCG to the cells

there is a chance to restore balance to the cells undergoing oxidative stress. The antioxidants can restore equilibrium to the imbalance of oxidants and antioxidants. (Zhang et al, 2009)

Salidroside is a glycoside from the Rhodiola rosea plant. A glycoside is when a molecule in which a sugar is bound to another group by a glycoside bond. Glycosides place vital role in storing chemicals in living organism. Salidroside has been known for antioxidant activity which could relate to EGCG and Ginkgo biloba. The abundance of antioxidants can strengthen the cells and deter them from undergoing extensive stress from oxidation. Also Salidroside has been to show decreased ROS production which is a product of oxidative stress. (Xiao et al, 2014)

Cao et al (2007) studied the application of Ginkgo biloba extract and ginseng on the rate of sarcopenia on a C. elegans model. Sarcopenia is the loss of skeletal muscles which is a neurodegenerative disease similar to HD in that both diseases cause the loss of neuron cells. The groups were separated in control and transgenic which was PD4251 strain along with treating all transgenic worms with the Ginkgo. They were able to run a fluorescence microscopy assay by embedding the live worms onto 2% agarose pads and GFP expression was conducted using the fluorescent microscope. The results showed that when the PD4251 worms that were exposed to Gingko had a longer lifespan when under the extreme stress from the oxidation. They also ran a motility assay where the worms were tapped with a platinum wire and then the number of body bends were compared to the pharyngeal pumping rate using the GFP expression. There was a slight increase in the number of body bends performed when the transgenic worms were exposed to Gingko. This could potentially be explained by the anti-oxidation properties subsidizing the severe effects the strain is putting on the worms.

Zhang et al (2009) studied the effects of EGCG on ROS production on a *C. elegans* using control and CL2070 worms. The worms were exposed to 300 millimolar for hour to prepare the worms for the rate of ROS production. The worms were then transferred to a microplate and then examined under a microplate reader to understanding the expression of the worms. Similar to Cao et al (2007), Zhang et al (2009) used GFP expression to examine the effects of the stress induced worms on a microscopic level. The transgenic worms exposed to EGCG under normal

conditions showed a decrease in ROS production which could be suggested by EGCG's anti-oxidation properties restoring equilibrium to the cell.

Xiao et al (2014) hypothesized that salidroside would protect the *C. elegans* from the harmful effects of oxidative stress and polyQ aggregation. They ran a paraquat assay which was when 75 microliters of paraquat was exposed to the worms to induce them into oxidative stress. They also measured ROS production very similarly to the methodology of Zhang et al (2009). The results showed that the salidroside did subdue the rate of oxidation. This could support that the salidroside was aiding the worms under the extensive high levels of stress. Lastly, the results also showed that the salidroside slightly increased lifespan of the transgenic worms exposed to salidroside.

Based on past literature, it is suggested that Gingko Biloba extract, EGCG, and Salidroside have the ability to reduce ASH neuronal death and the rate of oxidative stress along with increasing motility. A motility, oxidative stress, and chemotaxis assay will be performed to try and prove this theory. Statistical analysis will be performed using IBM SPSS $p<0.05$ after the data is collected.

B. Research Question(s), Hypotheses, and Expected Outcomes

Research Question:

Will Salidroside, Ginkgo Biloba extract, and Epigallocatechin gallate increase motility along with decreasing the rate of oxidative stress and ASH neuronal death in an HD model of *C.elegans?*

Hypotheses:

Alternate Hypothesis: Based off of the research performed by McColl et al (2007) and Xiao et al (2014) it is hypothesized that 50μM and 150μM Salidroside, 100μg and 200μg Ginkgo Biloba extract, and 1.0μg and 5.0μg Epigallocatechin gallate will increase motility, decrease the rate of oxidative stress, and improve ASH neuron function in a HD model of *C.elegans.*

Null Hypothesis: Salidroside, Gingko Biloba extract, and Epigallocatechin gallote will not affect nor improve motility, ASH neuron function, and rate of oxidative stress in an HD model of *C.elegans.*

Expected Outcomes:

When the worms are exposed to 100μg and 200μg Gingko Biloba, 1.0μg and 5.0μg EGCG, and 50μM and 150μM Salidroside the effects of the strains are expected to be lessened. For the motility assay the worms are expected to have increase in locomotion after a nose tap stimulation. For the chemotaxis assay, the transgenic worms exposed to the variables are expected to demonstrate improvement of their damaged ASH neurons, but not complete recovery. In respect to the oxidative stress assay, the naturally forming compounds are expected to decrease the rate of oxidative stress.

C. Procedures:

- **Protocols:**

Sterile Techniques

Throughout the entirety of the experiment all tools and equipment will be sterilized prior to use. All work areas will be disinfected with 10% bleach before and after any work is conducted. An apron, googles, and gloves will worn at all necessary times.

Caenorhabditis elegan strains

C.elegans N2, HA759, and HA659 will be donated from the Caenorhabditis elegans Genetic Center located in Minnesota.

HA759 worms will be examined under the fluorescence microscope specifically the Htn-Q150 which exhibits the polyglutamine aggression. HA759 is already tagged with GFP while HA659 is not. Both strains HA759 and HA659 both exhibit an increase in ASH neuron

death. The higher the rate of ASH neuron death correlates to an increase of progression of neurodegenerative diseases like HD. (Caenorhabditis Genetic Center, 2019)

Preparation of NGM plates

The nematode growth medium (Carolina Biological) will be microwaved until all solid agar becomes liquid. The top of the bottle will be unscrewed and loosely placed on top of the bottle. The agar bottle will be placed in the microwave for one minute at the highest level and closely monitored. The increments of time in the microwave will be shortened as more of the agar turns to liquid. Once the agar is completely melted, it will be removed from the microwave and transferred to the fume hood. Agar will be poured into sterile petri dishes approximately halfway with agar (or desired amount will be pipetted). Once all of the agar is poured, the petri dishes will be left to solidify for approximately ten to fifteen minute. NGM agar plates will either be used immediately or sealed with parafilm for later use. The empty bottle NGM bottle will be thrown out in the autoclave disposal bag and the counter will be wiped down with 10% bleach in order to disinfect the work area. (Caenorhabditis Elegans Culture Kit, 2000)

Preparation of *E.coli* OP50

The mouth of the nutrient broth tube will be sterilized. The cap will be loosely placed back onto the mouth of the test tube and then it will be placed on the rack. The cap of the *E.coli* (OP50) broth culture will be loosened and the mouth of the tube will be sterilized using an open flame. Approximately one milliliter of inoculum will be measured and then 0.2 milliliters of the *E.coli* will be distributed into every single test tube. After usage the serological pipet will be disposed of in the autoclave bag. All the mouths of the tubes will be sterilized with a flame prior to sealing. All cultures will be labeled and dated and will be incubated at thirty seven degrees Celsius for twenty four hours. (Caenorhabditis Elegans Culture Kit, 2000).

Administration of *E.coli* (OP50)

First, the *E.coli* OP50 culture will be taken out of the incubator and placed under the fume hood. A sterile cotton applicator will be placed in the inoculum and will be lightly spread

across the agar. The NGM plates will be sealed with parafilm and will be placed upside down in a sterile area. The *E.coli* must be on the agar for at least 24 hours before chunking worms onto the plate to establish a lawn. (Caenorhabditis Elegans Culture Kit, 2000)

Chunking Worms

The *C. elegans* will be identified under the microscope prior to chunking. The required supplies will include sterile scalpel, a beaker, 70% isopropyl alcohol, 10% bleach and a Bunsen burner. The Bunsen Burner will be turned on and once there is a flame the scalpel will be sterilized via flame sterilization. The scalpel will then be tested on the agar to make sure it is not too hot before cutting the worms out. Then, a 1 centimeter block will be cut and will be transferred onto an NGM plate with *E.coli* OP50. All petri dishes will be labeled and parafilmed. The new *C.elegans* cultures will be kept in an area with minimum to no air drafts and a constant temperature. (Caenorhabditis Elegans Culture Kit, 2000)

Age Synchronization of *C.elegans*

A plate will be used that has had worms growing for about 2-3 days in order to increase the number of worms present. Once there are substantial worms on the plate, 5 mL of M9 buffer will be added onto the plate and mixed. The solution will then be transferred to a microcentrifuge tube. The worms/M9 solution will then be centrifuged for 1 minute at 14,000 rpm. At this point a worm pellet should appear on the bottom of the tube. M9 buffer will be pipetted out and will be replaced with 20% alkaline hypochlorite to kill all the adult worms. The remaining solution will be centrifuged again for 1 minute at 14,000 rpm. The 20% alkaline hypochlorite will then be pipetted out, being careful to not disturbe the worm pellet, and will be replaced with the M9 buffer and centrifuged again at 14,000 rpm. This step will be repeated once more. Lastly, about 7mL of new M9 buffer will be added. The worm pellet will be vortexed or mixed in order to suspend the pellet. The tube will be placed onto an orbital shaker for 24 hours at a speed of 120 rpm and the resulting liquid will be poured onto seeded plates. (Sulston and Hodgkin, 1998)

Worm Picking

A Bunsen Burner, 28 gauge platinum wire, and a pasteur pipettes will be used in order to make a worm pick. A segment of the platinum wire will be placed halfway into the pasteur pipettes. The end of the pipette will be placed into the flame and meld the two materials using forceps. Once the worms are located under the microscope, the worm pick will be sterilized using the Bunsen Burner. The pick will be gently scraped along the surface of the agar while trying not to break the agar. Once worms are on the pick, the petri dish that has no worms on it will be opened and tip of the platinum wire will be gently slid onto the NGM surface. The pick will be sterilized every time the worms are transferred to prevent cross contamination. (Stiernagle, 2006)

Oxidative Stress Assay

A major cause of HD is the extreme levels of oxidative stress that the cells undergo. For this assay the worms will be put under extreme levels of oxidative stress and then the naturally forming compounds will be applied to see if this affects the rate of oxidative stress. The worms will be pre-exposed to 0.1M 20% hydrogen peroxide (H_2O_2) in order to reach a state of oxidative stress. The hydrogen peroxide will be applied on top of the agar and the worms will remain in the hydrogen peroxide for the duration of the experiment. The worms will have to be age synchronized to the L1 stage and exposed to Gingko Biloba, EGCG, and Salidroside for at least twenty four hours as previously stated Zhang et al (2009). The age sync is 24 hours because that is the time it takes to complete embryonic development. The data will be collected by counting the number of live or dead nematodes every 12 hour interval. Worms will be pronounced dead if they fail to perform stimulation from the worm pick (Xiao et al, 2014).

Fluorescence Analysis of *C.elegans*

GFP will be used after the oxidative stress assay is run in order to examine the harmful effects of hydrogen peroxide on a microscopic level. The 100μg/mL and 200μg/mL Gingko

Biloba, 1.0μg/mL and 5.0μg/mL EGCG, and 50μM and 150μM Salidroside worms will be imaged on a five day basis to see a progression and compare photos. The worms will be exposed to the oxidative stress and images will be taken on days 1, 3, and 5. HA759 and HA659 worms will undergo fluorescence microscopy (250x) with excitation at 485 nm and emission range of 530 nm. Photographic pictures will be taken to quantify the results of the oxidative stress assay.

Motility Assay

Since HD is a neurodegenerative disease so a motility assay will be able to see if the locomotion improves after exposure after naturally forming compounds like Ginkgo Biloba, Salidroside, and Epigallocatechin gallate. First the worms will be viewed under a dissecting microscope and the Snap Zoom attachment will be used to take videos of the worms. A worm pick will be sterilized via Bunsen Burner flame sterilization. After sterilization, the nose of the desired *C.elegans* worm will be touched with the sterilized worm pick. By prodding the nose of the worm, this will stimulate immediate locomotion and the worm will conduct a backward body bends. The number of body bends in 60 seconds will be recorded and put into Image J. The worms will also be exposed to the desired chemicals and will be tested after 24 hour exposure. The average number of body bends per variable will be graphed and compared (Worm Book, 2005).

Image J

Image J will be the computer program used to further analyze the motility assay. A thrashing quantification will run after the motility assay takes place. Once the worms under the microscope Snap Zoom can be used to record videos off the worms after stimulation. The videos will be uploaded into the Image J software. Once the videos are uploaded the computer program specifically counts the number of body bends using specific algorithms. (Gallagher, 2003).

Image J will also be used for quantification of the GFP photographs that will be taken during the Fluorescence Microscopy assay. This will quantify the fluorescence observed in the

pictures which will correlate to the rate of ASH neuronal death in primarily the head and tail of C.elegans.

Chemotaxis Assay

The chemotaxis assay will be able to test the function of the ASH neurons present in the worms. The main function of the ASH neurons is for the worms to be able to identify different odors and this affects their attractance. When the transgenic worms are exposed to oxidative stress, this may affect the function of their ASH neurons. By exposing the worms to 1.0 and 5.0 μg/mL, salidroside, 100 and 500 μg/mL Ginkgo biloba, and 50 and 150 μM Epigallocatechin gallote the function of the ASH neurons may be repaired (Margie, 2013).

To make a chemotaxis plate, the petri dish will be divided into four quadrants. First, the underside of the petri dish will be broken into four quarters using a thin marker. Next, the center of the lines will be marked with a 0.5 cm circle around the origin of the lines. Then, a label will be placed in each quadrant - "T" for test or "C" for control, making sure that the letters are equidistant from each other and are 2cm from the origin. Before starting the assay, the worms will be age synced to the young adult stage. Next, 2 mL of S basal will be pipetted onto the petri dish with worms on it. Using a pipette, S basal solution with worms in it will be removed and will be transferred into a micro centrifuge tube. The solution of S basal and worms will be centrifuged for about 10 seconds on maximum speed. Using a pipette, the S basal solution will be removed and care will be taken to not disturb the worm pellet. New S basal solution will be added into the tube and the previous steps will be repeated three times. 2 mL of the worms will then be pipetted onto the chemotaxis plate and the assay will begin immediately after transferring. (Margie, 2013).

100mL of water and 10g of sodium chloride will be mixed to create the Test solution. The control solution will be the NGM agar with no additive. The Test solution will be pipetted into the "T" quadrants and the control solution into the "C" quadrants. Then, the lid of the petri dish will be replaced in order to prevent contamination. After sixty minutes, the plate with the worms will be exposed to 4 degrees Celsius. After exposure, the worms will be let to settle in order to

allow them to mobilize. The worms will be examined under the microscope and the amount of worms in each quadrant will be counted. At the conclusion of the test, 10% bleach will be administered to the petri dish, parafilmed, and will be discarded into the garbage (Margie et al, 2013).

To calculate the data, the chemotaxis index will be used. The chemotaxis index is (# Worms in Both Test Quadrants - # Worms in Both Control Quadrants) / (Total # of Scored Worms). A +1.0 will correlate to maximum attraction and all of the worms will have moved to quadrants with the chemical. An index of -1.0 will correlate to there being a maximum repulsion, which will suggest that the function of the ASH neurons decreased (Margie et al, 2013).

- **Risk and Safety**

1. *Human Participants Research: N/A*
2. *Vertebrate Animal Research: N/A*

3. Potentially Hazardous Biological Agents:

As per ISEF rules and guidelines, this study involves BSL-1 organisms that are exempt from prior SRC review and require no additional forms: (ISEF Rulebook Page 16, section 2f.)
As such, all organisms outlined below will be listed on ISEF Form 3.

Organism Name: ***Escherichia coli* OP50***

**E. coli OP50 will be used as a food source for the C. elegans*

a. Source of Organism: *Caenorhabditis Genetics Center (CGC)*
   BSL assessment determination: BSL- 1

b. Safety precautions: The student researcher will be trained by the designated supervisor in all safety aspects associated with working with bacteria, sterile technique and proper handling and disposal of the bacteria. The designated supervisor will directly supervise the student researcher when working with the bacteria. Goggles, lab apron and nitrile gloves will be worn during experimentation. Prior to use, all surfaces will be wiped with 10% bleach solution. All surfaces will also be wiped down with 10% bleach after experimentation.

Methods of disposal: 10% bleach will be used to to kill the bacteria. 10% bleach will be incorporated onto the NGM agar plate and the plate will be sealed with parafilm and disposed of.

Organism Name: **N2** ***Caenorhabditis elegans***

a. Source of Organism: *Caenorhabditis Genetics Center (CGC)*
BSL assessment determination: BSL- 1

b. Safety precautions: The student researcher will be trained by the designated supervisor in all safety aspects associated with working with *C. elegans* and bacteria, sterile technique and proper handling and disposal of the bacteria and c. elegans. The designated supervisor will directly supervise the student researcher when working with the bacteria. Goggles, lab apron and nitrile gloves will be worn during experimentation. Prior to use, all surfaces will be wiped with 10% bleach solution. All surfaces will also be wiped down with10% bleach after experimentation.

Methods of disposal: 10% bleach will be used to to kill the bacteria and *c. elegans*. 10% bleach will be incorporated onto the NGM agar plate and the plate will be sealed with parafilm and disposed of.

Organism Name: **HA759** ***Caenorhabditis elegans***

a. Source of Organism: *Caenorhabditis Genetics Center (CGC)*
BSL assessment determination: BSL- 1

b. Safety precautions: The student researcher will be trained by the designated supervisor in all safety aspects associated with working with *C. elegans* and bacteria, sterile technique and proper handling and disposal of the bacteria and c. elegans. The designated supervisor will directly supervise the student researcher when working with the bacteria. Goggles, lab apron and nitrile gloves will be worn during experimentation. Prior to use, all surfaces will be wiped with 10% bleach solution. All surfaces will also be wiped down with10% bleach after experimentation.

Methods of disposal: 10% bleach will be used to to kill the bacteria and *c. elegans*. 10% bleach will be incorporated onto the NGM agar plate and the plate will be sealed with parafilm and disposed of.

Organism Name: **HA659** ***Caenorhabditis elegans***

a. Source of Organism: *Caenorhabditis Genetics Center (CGC)*
BSL assessment determination: BSL- 1

b. Safety precautions: The student researcher will be trained by the designated supervisor in all safety aspects associated with working with *C. elegans* and bacteria, sterile technique and proper handling and disposal of the bacteria and c. elegans. The designated supervisor will directly supervise the student researcher when working with the bacteria. Goggles, lab apron and nitrile gloves will be worn during experimentation. Prior to use, all surfaces will be wiped with 10% bleach solution. All surfaces will also be wiped down with10% bleach after experimentation.
Methods of disposal: 10% bleach will be used to to kill the bacteria and *c. elegans*. 10% bleach will be incorporated onto the NGM agar plate and the plate will be sealed with parafilm and disposed of.

4. Hazardous Chemicals, Activities & Devices:

Ginkgo Biloba (100 and 200 µg/mL (DMSO solvent), 200mL):

- Ginkgo Biloba is categorized as a non-hazardous substance or mixture by Sigma Aldrich.
- When working with this substance all matters will be supervised by an adult.
- An apron, nitrile gloves, and goggles will be worn at all times when working with Gingko Biloba. Solutions will be stored in a locked flammables cabinet when not in use.
- To dispose of Ginkgo Biloba, the excess solution will be offered to licensed disposal company for disposal.

Epigallocatechin gallate (EGCG) (1.0µg/mL and 5.0µg/mL (water solvent), 200mL)

- EGCG is categorized as a non-hazardous substance or mixture.
- A supervised adult will be in the lab at all times when working with these chemicals.
- An apron, nitrile gloves, and goggles will be always be worn when working with this chemical.
- To dispose of this, the excess solution will be offered to a licensed disposal company.

Salidroside (50μM and 150μM (DMSO solvent), 200mL):

- Salidroside is categorized as a non-hazardous substance or mixture. Salidroside may cause eye irritation when coming in contact with the eye.
- When working with this substance all matters will be supervised by an adult.
- Goggles, nitrile gloves, and an apron will be worn at all times. If the chemical happens to get into the eye, the eye will be immediately rinsed out at the eye wash station located in the lab. Solutions will be stored in a locked flammables cabinet when not in use.
- To dispose of this, the excess solution will be offered to a licensed disposal company.

Hydrogen Peroxide (20%, 50mL)

- Hydrogen Peroxide is categorized as being harmful if swallowed. Hydrogen peroxide may cause severe skin burns and eye damage. It is considered to be corrosive to the skin.. It may cause respiratory irritation and is considered to be toxic to aquatic life and harmful to aquatic life with long lasting effects.
- All handling and use of hydrogen peroxide will be supervised by an experienced teacher or mentor.
- Tightly fitted eye goggles will be worn. Nitrile gloves (0.11mm) will be worn and inspected before use. A lab apron will be worn. When not in use, hydrogen peroxide will be stored in a locked corrosives cabinet. Care will be taken to avoid breathing dust/fume/mist/vapours/spray.
- This chemical will not be released into the environment. To dispose of this, the excess solution will be offered to a licensed disposal company.

Ethanol (40%, 50mL):

- Ethanol is considered a hazardous substance that is flammable and when it comes in contact with the eye, it can cause irritation or eye damage
- Adult supervision will be at all times during the use of this substance.
- Goggles, nitrile gloves, and a lab apron will be worn at all times. Ethanol will be kept in the locked flammable cabinet when not in use.
- To dispose of this, the excess solution will be offered to a licensed disposal company.

M9 Buffer (100 mL)

- M9 buffer is categorized as short term aquatic hazardous substance.
- A teacher will be present when working with this chemical.
- When working with this substance goggles, nitrile gloves, and an apron will be worn at all times.
- To dispose of this, the excess solution will be offered to a licensed disposal company.

70% Isopropyl Alcohol (100 mL)

- Isopropyl alcohol is considered a highly flammable substance along with having the ability to cause eye irritation if not used probably.
- A teacher will be present at all times in the lab when working with this substance.
- When using Isopropyl alcohol goggles, an apron, and latex gloves will be used. Isopropyl alcohol will be stored in a flammable cabinet when not in use.
- To dispose of this, the excess solution will be offered to a licensed disposal company.

DMSO (400mL): *will be used as a solvent for Ginkgo Biloba and Salidroside*

- DMSO is categorized as a combustible liquid.
- A teacher will be present at all times in the lab when working with this substance.
- Nitrile gloves, goggles, and lab aprons will be worn at all times. It should be kept away from any open flames or hot surfaces. DMSO will be stored in a locked flammable cabinet when not in use. If a person breathes in vapors or mist from the DMSO, the person would be immediately moved into a fresh open space and if unconscious artificial respiration should be performed.
- To dispose of this, the excess solution will be offered to a licensed disposal company..

Sodium Chloride(5g/100mL water)

- Sodium chloride is categorized as a non-hazardous substance or mixture.
- A teacher will be present at all times in the lab when working with this substance..
- Goggles, nitrile gloves, and an apron will be worn when working with chemicals.
- For disposal, the surplus and non-recyclable solutions will be distributed to a licensed disposal company.

Alkaline Hypochlorite (20% 50mL):

- Alkaline Hypochlorite a category 2 skin irritation, category 2A eye irritation, category 3 acute aquatic toxicity, and category 3 aquatic life toxicity.
- A teacher will be present at all times in the lab when working with this substance.
- Goggles, nitrile gloves, and an apron will be worn when working with chemicals. Exposure to the skin and eye contact will be avoided at all times.. If skin irritation occurs medical attention will be sought out as soon as possible.
- For disposal, the surplus and non-recyclable solutions will be distributed to a licensed disposal company.

Autoclave

- If the autoclave is open prior to depressurization, there is a risk of getting burned by hot steam.
- A teacher will present when this piece of machinery is being used.
- When working the autoclave the latches will remain closed at all times. Autoclave gloves and lab apron will be worn when removing hot (and sterile) solutions and items from the autoclave to prevent burns.

Bunsen burner

- A teacher will be present when using this piece of equipment.
- An apron, googles, and nitrile gloves will be worn at all times when using the Bunsen burner. Also, long hair will be tied back along with pulling back any baggy sleeves/clothing pieces. The teacher will be the only person with permission to turn on the gas to allow the Bunsen burner to work.
- Any tubes connecting the gas hose to the Bunsen Burner that appear to have holes or damage to them will be discarded.

Centrifuge

- If the centrifuge is opened and touched prior to stopping, there is a risk of damaging fingers/hands.
- A teacher will be present when this piece of machinery is being used.
- Also the centrifuge will only be opened when the machine comes to a complete stop so the solutions can be safely retrieved.

Scalpel

- If used incorrectly, there is a risk for getting cut on the sharp scalpel.
- Also a teacher will be supervising when using this tool for safety precautions.
- The scalpel will be kept in its protective case when it is not being used. A scalpel will be kept in a lab cabinet with its' case on at all times.
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- **Data Analysis**

Data from the motility assay and oxidative stress assay will be averaged and graphed. Descriptive statistics and significance will be measured and determined using IBM SPSS v.25. A one-way ANOVA followed by a post hoc scheffe, $p<0.05$ will be used to measure statistical significance. Data from the chemotaxis assay will undergo a T-test and the chemotactic indices will be graphed.

D. **Bibliography**

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Project Summary:

NO ADDENDUMS EXIST