Research Paper

The Effect of Ginkgo Biloba, Salidroside, and Epigallocatechin Gallate on ROS Production, Motility, Fertility, and Neural Activity on a Huntington's model of C. elegans

By: Spyrithoula Xenophontos and Kaitlyn Clarke

Abstract

According to the Huntington's Disease Society of America (HDSA) in 2018, there are approximately 300,000 Americans with Huntington's disease (HD and 200,000 Americans at risk of inheriting HD. HD is distinguished by the progressive deterioration of nerve cells in the brain. Ginkgo Biloba, Epigallocatechin gallate (EGCG), and Salidroside are all antioxidants known to possess neuroprotective properties and anti-oxidation properties. Based off the work of Zhang et al and Cao et al, it was hypothesized that 50µM and 150µM Salidroside, 100µg and 200µg Ginkgo Biloba, and 1.0µg and 5.0µg EGCG would increase motility, decrease ROS production, and decrease ASH neuronal death in a Huntingotn's model of C.elegans.All strains were age synchronized to the L1 stage. A motility assay was run where all worms were exposed to the antioxidants over a four day period. The worms received stimulation daily from a platinum worm pick and the number of body bends were uploaded to Image J for quantification. For the chemotaxis assay worms were centrifuged and pipetted into the center of a four quadrant divided petri dish with NaCl acting as an attractant. For the oxidative stress assay the *C.elegans* were pre-exposed to antioxidants for 24 hours then exposed to Hydrogen Peroxide (H_20_2) for 24 hours. On Days 1,3,5 the HA759 worms were photographed at 250x magnification and uploaded to Image J for CTCF. A one-way ANOVA followed by a post-hoc Scheffe test ($p<0.05$) using IBM SPSS version 25 was performed. The average number of body bends for the HA759 was 17.4 body bends, while the 150 µM salidroside exhibited the highest average of 35 body bends. In the chemotaxis assay the 50µM salidroside showed a much higher attractance of 0.1while HA659 had -0.54 attractance. The results supported the alternate hypothesis that the neuroprotective properties of the three antioxidants remediated the effects of the HD strains.

Introduction

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 effective because they have 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 are HD and juvenile HD. Both are very similar, but are different based off of the 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 and cognitive disorders. HD is a genetic disease because the HD gene can be inherited onto the carriers' offspring. HD is an autosomal dominant disorder which 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 (H.C. et al, 2014). 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 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)

Ginkgo 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 Ginkgo 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 sugar molecule is bound to another group by a glycosidic bond. Glycosides have a vital role in storing chemicals in living organisms. 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 shown to decrease 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. They found that there was a slight increase in the number of body bends performed when the transgenic worms were exposed to Gingko. Results of the GFP assay showed that transgenic worms had a longer lifespan when under the extreme stress from the oxidation. 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. Xiao et al (2014) hypothesized that salidroside would protect the *C. elegans* from the harmful effects of oxidative stress and polyQ aggregation. The results showed that the salidroside slightly increased the lifespan of the transgenic worms exposed to salidroside and decreased ROS production.

The purpose of this research is to investigate if Ginkgo Biloba, Salidroside, and EGCG can remediate the effects of the HA759 and HA659 strain. Based off of the research performed by McColl et al (2007) and Xiao et al (2014) it is hypothesized that Salidroside, Ginkgo Biloba extract, and Epigallocatechin gallate will increase motility, increase the rate of reproduction, decrease the rate of oxidative stress, and improve ASH neuron function in a HD model of *C.elegans.* The null hypothesis is that Salidroside, Ginkgo Biloba extract, and Epigallocatechin gallate would not affect or improve motility, fertility, ASH neuron function, and rate of oxidative stress in an HD model of *C.elegans.*

II. Methodology

In both phases, the HA759 strain which expresses the httq-150 fluorescent (GFP) protein and HA659 strain which induces ASH neuronal death were obtained from the Caenorhabditis Genetic Center.

A motility assay was run where the N2, HA659, and HA759 strains were exposed to six different concentrations of Ginkgo Biloba, Salidroside, an EGCG. Then the worms received stimulation and the number of body bends performed was recorded and the videos were uploaded to Image J. Then the number of average body bends were averaged and graphed.

Secondly, an oxidative stress assay was run to examine if the antioxidants could decrease the rate of ROS production. The worms were age synced to the L4 stage and then exposed to hydrogen peroxide for twenty four hours. After 24 hours of exposure to hydrogen peroxide, the number of live worms were recorded. Worms were pronounced alive if they reacted to stimulation by a worm pick. GFP analysis was also performed for the HA759 plates.

A fertility assay was performed which analyzed the rate of reproduction of the *C.elegans* over the span of three days. Worms were age synchronized and picked onto plates over the course of three days. Each day the number of worms under the microscope was recorded and graphed.

Lastly, a chemotaxis assay was run to analyze ASH neuronal death. The main function of the ASH neurons is for the worms to be able to identify different odors and this affects their attractance. Now when the transgenic worms are undergoing severe amounts of oxidative stress this may affect the function of their ASH neurons. By exposing the worms to 1.0 and 5.0 micrograms salidroside, 100 and 200 micrograms Ginkgo biloba, and 50 and 150 micro molar Epigallocatechin gallate the function of the ASH neurons may be repaired. The plate was setup in a four quadrant setup and the worms were exposed to different solutions to see if the antioxidants could remediate and decrease ASH neuronal death.

Sterile Techniques

Throughout the entirety of the experiment all tools and equipment were kept as sterile as possible. All work areas were disinfected with 10% bleach before and after any work was conducted. An apron, goggles, and gloves were worn at all necessary times.

Preparation of NGM Plates

Before melting the NGM from Carolina Biological, the cap was unscrewed and loosely placed on the bottle. Then the agar was melted for decreasing one minute time increments and was closely monitored. Once the agar has turned to a liquid, it can be removed from the microwave and transferred to the fume hood. The necessary number of petri dishes were taken out and the petri dishes were filled halfway with agar. It takes approximately ten to fifteen minutes in order for the agar to completely solidify. The empty glass bottle was disposed of (Caenorhabditis Elegans Culture Kit, 2000).

Ginkgo Biloba, EGCG, and Salidroside Treatment

Ginkgo Biloba, EGCG, and Salidroside have the ability to remediate the effects of oxidative stress and ASH neuronal death due to the neuroprotective and antioxidant properties. Ginkgo Biloba was added to the agar at concentrations of 100 mg, 200 mg, 1.0 μg EGCG, 5.0μg EGCG, 150μM Salidroside, and 50μM Salidroside.

Preparation of E.coli OP50

The cap of the nutrient broth tube was loosened and sterilized using the flame produced by a Bunsen Burner. This tube was then placed back on the rack. Then the cap of the E.coli OP50 broth culture was loosened and sterilized using flame. 0.2 milliliters of the E.coli broth was administered into 1 milliliter of inoculum using a serological pipet. The mouths of both tubes were exposed to the flame again and the serological pipet was properly disposed of. All cultures were labeled, dated, and incubated at thirty seven degrees Celsius for twenty four hours. (Caenorhabditis Elegans Culture Kit, 2000).

Administration of E.coli OP50

The *E.coli* tube was taken out of the incubator and transferred to the fume hood. Sterile cotton applicators and non-seeded plates were also placed in the fume hood. The cotton applicators were placed in the inoculum and then were lightly spread across the agar. The cotton spreaders were disposed in the autoclave disposal bag after usage. The plates were then placed upside down in a sterile area (Caenorhabditis Elegans Culture Kit, 2000).

Chunking Worms

The worms had been previously identified under the microscope. The sterile scalpel, a beaker, isopropyl alcohol, and a Bunsen burner were required. The Bunsen burner was first turned on and the scalpel was sterilized using the alcohol and the flame. The scalpel was then tested on the agar before cutting from the plates with worms. 1 centimeter blocks were cut out and the blocks were transferred onto the E.coli plates. All new plates were transferred and the new C.elegans cultures were kept in well ventilated areas at constant temperature.

Age Synchronization of *C.elegans*

A plate was used that contained worms that had been growing for about 2-3 days. Once there were substantial worms, an M9 buffer was pipetted onto the plate and then this solution was pipetted into a centrifuge tube. The worms were then centrifuged for 1 minute and a worm pellet appeared on the bottom of the tube. The M9 buffer was replaced for 20% alkaline hypochlorite to kill all the adult worms and was centrifuged again for 1 minute. The 20% alkaline hypochlorite was taken out without disturbing the worm pellet and replaced with M9 buffer again. The worms were then centrifuged again at the same settings. This step was then

repeated again. Lastly, 7mL of new M9 buffer was added and the tube was tapped in order to suspend the pellet. Then the tube was placed on a shaker for 24 hours and the liquid was poured on seeded plate the next day (Sulston and Hodgkin, 1998).

Motility Assay

Since HD is a neurodegenerative disease, a motility assay was run to investigate locomotive response after the *C.elegans* were exposed to Gingko Biloba, Salidroside, and Epigallocatechin gallate. The worms were located under the microscope and the Snap Zoom attachment was placed on the microscope in order to take videos of the worms. The platinum worm pick was sterilized using the flame from the Bunsen Burner on the lowest setting. Once the pick was sterilized, the worm being stimulated was identified under the microscope. The nose of the chosen worm was tapped and the number of body bends performed was recorded in a thirty second video. These videos were uploaded to the Image J software and the average number of body bends per variable were graphed and analyzed (Worm Book, 2005).

Image J

Image J was the computer software program used to analyze the motility assay. Image J specifically counted the number of body bends using specific algorithms. The videos recorded with Snap Zoom were converted to AVI format and uploaded to the program. A thrashing quantification was run to quantify the number of complete body bends performed which is more accurate than the human eye (Gallagher, 2003).

Oxidative Stress Assay

For this assay the worms were put under high levels of oxidative stress and then exposed to all three naturally forming compounds. The worms were age synchronized to the L1 stage and exposed to Gingko Biloba, EGCG, and Salidroside for at least twenty four hours before exposure to the hydrogen peroxide (Zhang et al, 2009). The worms were then pre-exposed to 0.1M 20% hydrogen peroxide (H202) for twenty four hours in order to have reached a state of oxidative stress. The data was collected by counting the number of live and dead nematodes every 12 hour interval. Worms were pronounced dead if they fail to perform stimulation from a platinum worm pick (Xiao et al, 2014).

Fluorescent Analysis of *C.elegans*

GFP analysis was performed after the oxidative stress assay was run in order to examine the harmful effects of hydrogen peroxide on a microscopic level. The worms were imaged on a seven day basis to see a progression and compare photos. Images were taken on day 1,3,5, and7. HA759 worms will undergo fluorescence microscopy. Photographic pictures were taken to quantify the results of the oxidative stress assay.

Phase 2 Methodology

Chemotaxis Assay

A chemotaxis plate was made by diving a petri dish into a four equidistant quadrant setup using a thin marker. Each quadrant was labeled “T” for test or “C” for control. The letters were marked equidistant from each other and 2cm from the origin. Before starting the assay the worms must be age synced to the young adult stage. 2 mL of S basal was pipetted onto a petri dish with worms on it. A pipette was used to suck up the S basal solution with worms in it into a

microcentrifuge tube.The worms were centrifuged for about 10 seconds on maximum speed. The S basal was suctioned out with a pipette without disturbing the worm pellet and new S basal was added back into the tube. These steps were repeated three times. 2mL of the worms were pipetted onto the center of the dish. The worms were used immediately after this procedure (Margie, 2013).

100mL water and 10g sodium chloride were mixed to create the Test solution. The control solution was just be the bare agar. The Test solution was pipetted onto the "T" quadrants and the control solution into the "C" quadrants. The lid of the petri dish was replaced in order to prevent contamination. For sixty minutes the worms were put in a 4 degrees Celsius incubator. After the worms came out of the incubator, the worms settled in order to allow them to mobilize. The worms were examined under the microscope and the number of worms in each quadrant were counted. (Margie et al, 2013).

III. Results

Motility Assay

The following graph presents the comparison of motility between the three strains of C.elegans used: N2, HA659, and HA759. The graph shows that the strains all have similar motility, as the average number of body bends for each strain is between 15 and 20 body bends. Though, the N2 has the highest at 18, HA759 second with 17.4, and

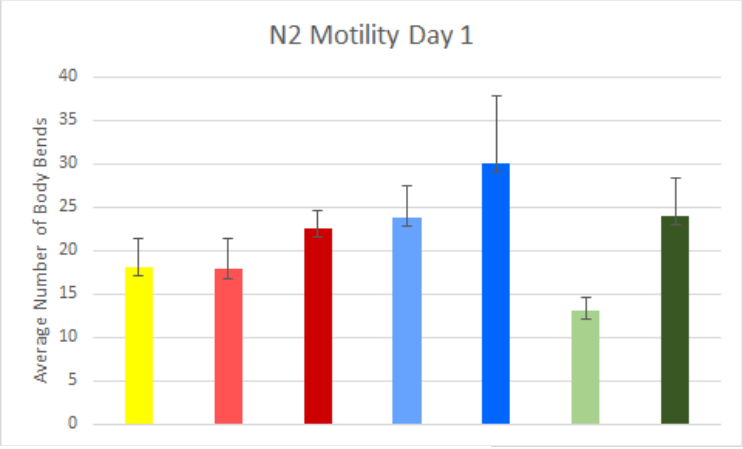


Figure 1- Control Motility Assay

HA659 the least motile at 16 body bends. This part of the assay tested the motility of each strain, in order to see their differences and take them into account as we conduct our antioxidant strain assays.

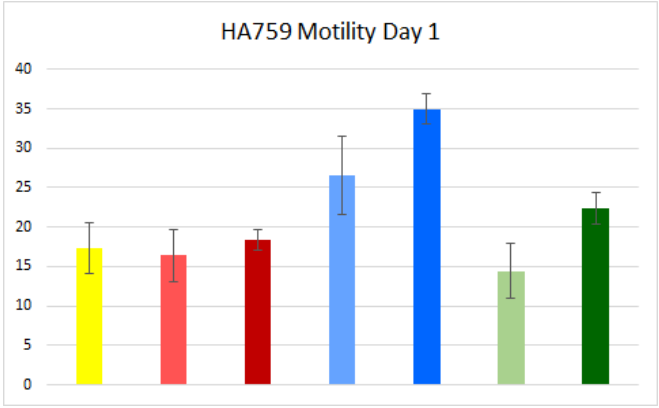


Figure 2- N2 Exposed To Variables

This graph shows the motility of the N2 antioxidant trial. Significance is denoted by the asterix on the 1.0 µg EGCG and the 150 µM salidroside groups. In all three antioxidants, EGCG, salidroside, and ginkgo biloba, the higher concentration exhibited higher motility. The antioxidant that demonstrated the highest motility was the salidroside, and the highest motility of all the variables was expressed by the 150 µg salidroside plate with an average number of body bends at 30. This is a significantly higher average than the EGCG and ginkgo biloba strains. The EGCG strain was the second most successful, as the 5.0 µg EGCG indicated a similar effect on motility as the 50 µg salidroside. Though, the 1.0 µg EGCG demonstrated the lowest average number of body bends at 13 body bends, with a significantly lower average than the N2 control trial which had an average of 18 body bends. Additionally, the EGCG showed the largest difference between the 1.0 µg and the 5.0 µg. The 200 µg ginkgo biloba resulted in similar effects on motility as the 5.0 µg EGCG and the 50 µM salidroside.

The HA759 variable trial has significance for 1.0 µg EGCG and 150 µM salidroside. In all three antioxidants, EGCG, salidroside, and ginkgo biloba, the higher concentration demonstrated higher motility. In this variable trial, the 1.0 µg EGCG and 100 µg G.Biloba groups were lower than the HA759 control average number of body bends. The 200 µg G.Biloba

barely affected the motility, with an average of 18.4, compared to the control at 17.4 body bends. The 5.0 µg EGCG affected the motility, but the salidroside had a greater effect. The 50 µM salidroside had an average of 26.6 body bends and the 150 µM had an average of 35 body bends.

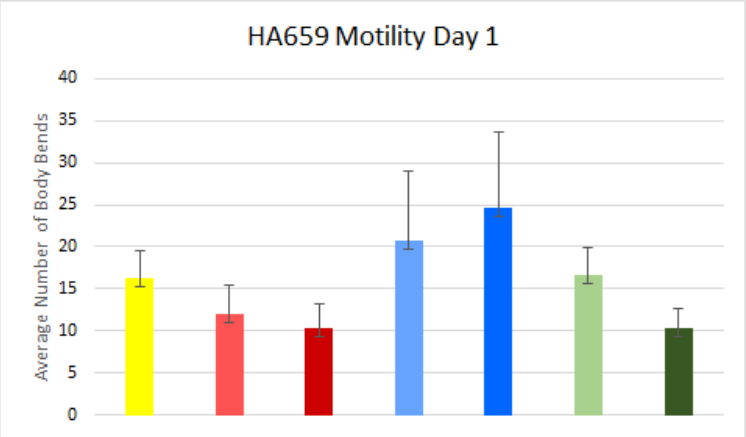
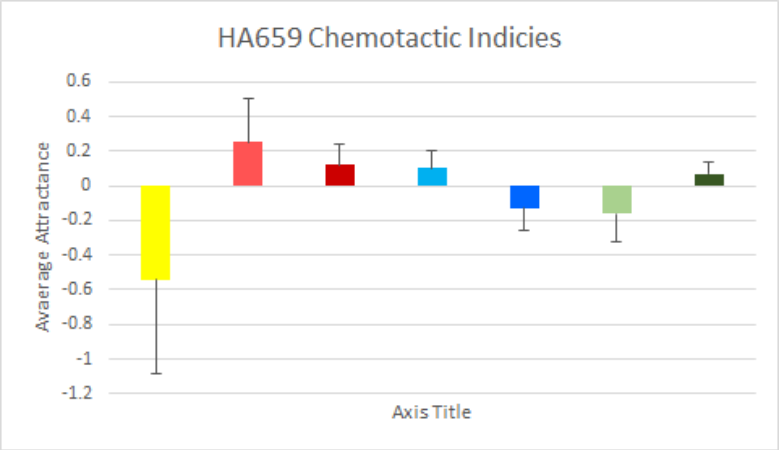


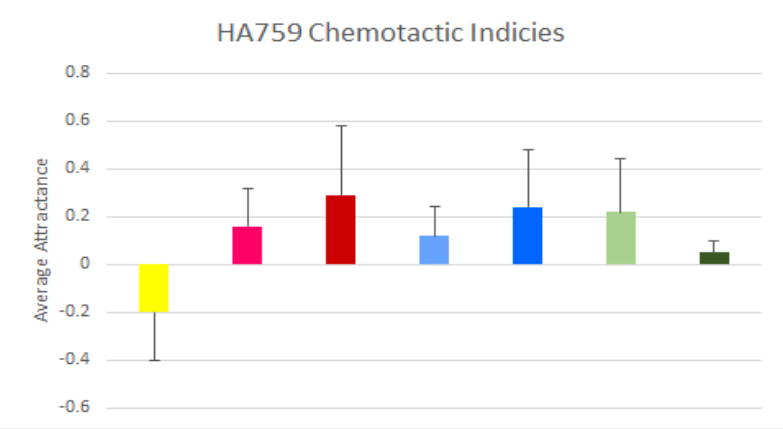
Figure 3- HA659 Exposed To Variables

The 1.0 µg EGCG, 5.0 µg EGCG, 100 µg G.Biloba, and 200 µg G.Biloba all showed significance. This trial was inconsistent with the other two in that in all three antioxidants, EGCG, salidroside, and ginkgo biloba, the higher concentration did not demonstrate higher motility. The 1.0 µg EGCG had a greater motility than the 5.0 µg and the 100 µg G.Biloba had a greater motility than the 200 µg. The average of the HA659 control was 16.2 body bends. The5.0 µg EGCG, 100 µg G.Biloba, and 200 µg G.Biloba all showed a lower average number of body bends than the control average. The 1.0 µg EGCG demonstrated an increase in the motility compared to the control trial. Though, the salidroside exhibited the greatest effect on the motility of the HA659 strain.

The salidroside demonstrated the greatest effect on motility, showing the highest average number of body bends in each strain's motility assay. As shown in figure one, the average number of body bends for N2 worms is 18, and in the antioxidant trial the highest average was 150 µM salidroside with an average number of body bends of 30. Though the 5.0 µg EGCG had a greater effect on motility compared to the 50 µM salidroside, the salidroside is still the most effective. This is likely due to the 5.0 µg EGCG being a greater concentration than the 50 µM salidroside. Additionally, the difference between the two averages is miniscule. The average for the HA659 worms was 16.2, and the HA659 variable trial showed that the salidroside had the greatest effect on motility, as it had the highest average of the trial and the greatest increase 16.2, with an average of 24.6 body bends. The average for the HA759 non-variable trial was 17.4 body bends, and again 150 µM salidroside exhibited the highest average of 35 body bends.



The chemotaxis assay did support the alternate hypothesis. This is because the average for the HA759 non-variable trial was 17.4 body bends, and again 150 µM salidroside exhibited the highest



average of 35 body bends. In the chemotaxis assay the 50μM salidroside showed a much higher attractance if 0.1while HA659 control was -0.54 attractance.

IV. Discussion

This experiment researched the anti-oxidation and neuroprotective properties of Ginkgo Biloba, Salidroside, and Epigolocatechin gallate on a Huntington's model of *C.elegans.* It could be postulated that the neuroprotective qualities protected the *C.elegans* allowing for them to be more motile along with surviving the exposure to hydrogen peroxide.

The results of the motility assay showed that when the transgenic worms were exposed to 150 μM salidroside, 150 μM salidroside, 200 μg Ginkgo Biloba, 100μg Ginkgo Biloba, 1.0 μg EGCG, and 5.0 μg EGCG there were increases in body bends and the data was significant following a One-Way Anova Post-hoc Scheffe $p<0.05$. Once the worms were exposed to the 150 μM salidroside for the HA659 worms averaged 30 body bends for 30 seconds while the average for the N2 worms was 18 body bends. The 5.0 μg EGCG did have a greater effect on motility compared to the 50 μM salidroside; however the salidroside is still the most effective because these groups averaged the highest in body bends. These results could be supported by the anti-oxidation properties of these compounds. When the transgenic worms are experiencing severe levels of oxidative stress, there is an imbalance of oxidants and antioxidants. By applying an abundance of antioxidants to the *C.elegans* there is a chance of restoring equilibrium to the cells. By restoring equilibrium to the cells of C.elegans, the effects of the HA759 and HA659 strains are being subdued allowing the worms to be more motile. This is similar to Zhang et al (2009) where they found that EGCG increased survival of the worms under extreme oxidative and heat stressors. To add, our data aligns with the findings of Cao et al (2007) where they found that

Ginkgo Biloba extract decreased muscle degradation in a sarcopenia model of *C.elegans* which allowed the worms to become more motile.

During the oxidative stress assay, the HA759 worms had more alive than dead worms post hydrogen peroxide exposure. There were 8 live worms and 5 dead worms counted. Even though there were more live worms it could be postulated that in the phase 2 when the worms are exposed to the naturally forming compounds like salidroside, there will be more of a difference between the live and dead number of worms. This would be similar to the findings of Xiao et al (2014), that found that the neuroprotective properties of salidroside were able to protect ASH neurons under high levels of oxidative induced by paraquat. Also the neuroprotective properties decreased the degradation and depletion of neurons exacerbating the effects of sarcopenia. Lastly, this just reinforces the point that once antioxidants are applied to an area of oxidative stress a temporary equilibrium can possibly be restored to the cell.

This experiment did have significant data, however, there were some limitations. For example, if the solutions weren't made often the chemicals would clump together in the bottle and then would clump in the agar. This was quickly resolved by making solutions more frequently to prevent the solution from becoming too old and preventing the chemicals from dispersing evenly. Secondly, when the worms were picked onto new plates mold would sometimes sporulate onto the plate within 24 hours. The solution to this problem was sterilizing with fire more frequently and having the pick only touch the agar once sterilized with fire. Lastly, the *E.coli* OP50 would become too dense to spread onto the agar. This was resolved by inoculating the *E.coli* more frequently in order to prevent the cultures from becoming dense.

V. Conclusion

In conclusion, the results of the locomotion and the controls of the oxidative stress assay suggest that EGCG, Salidroside, and Ginkgo Biloba remediated the effects of the HA759 and HA659 strains which restricted motility and increased neuronal deal. The results supported the alternate hypothesis which was that EGCG, Salidroside, and Ginkgo Biloba would increase motility and lessen the effects of oxidative stress. By exposing the worms to six different concentrations this allowed for more data to be analyzed and compared to see what the most beneficial solution was and which also allowed for the data to be statistically significant. Clearly, it could be postulated that all three of these naturally forming compounds increased motility and lessened the effects of oxidative stress for N2, HA659, and HA759 worms.

VI. Future Studies

In the future, the worms could be exposed to the naturally forming compounds for longer periods of time to see if this would remediate the effects of the transgenic strains. By having the exposure period be longer, this could allow for the balance between antioxidants and oxidants to be brought to a balance in a quicker and more efficient manner. However, having the worms exposed for longer times to the antioxidants could also have no effect as well.

Secondly, paraquat could be used for the oxidative stress assay to see if this induces the warms into a more intense level of oxidation. As done in previous literature the hydrogen peroxide could be inserted in the agar and this could possibly change the outcome of the worms and induce them into a higher level of oxidative stress. During the oxidative stress assay the worms could be checked every 12 hours instead of 24 as done in Xiao et al because this would allow for more accuracy in the data when comparing the controls to variables.

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