The Effect of Nonassociative Learning on Oxidative Stress in *Caenorhabditis elegans*: A Potential Application for Alzheimer's Disease Research

Gillian Patton

Spring Valley High School

Alzheimer's disease results in learning and memory deficits due to toxic changes in the brain caused by amyloid-β plaques and oxidative stress.[1] Oxidative stress is caused by the overproduction of free radicals, which are uncharged molecules containing an unpaired valence electron.[2] Nonassociative learning is a type of cognitive processing that only uses one stimulus instead of two related stimuli. Cognitive processing promotes neural growth and learning, while Alzheimer's inhibits it. The purpose of this study was to test how nonassociative learning impacts oxidative stress and thus, if it might be considered as a potential treatment option for Alzheimer's disease. It was hypothesized that the application of nonassociative learning would reduce the effects of oxidative stress on *C. elegans*, resulting in increased mobility and egg-laying. Mechanosensory, chemosensory, and novel environment habituation were used to increase mobility and egg-laying in *C. elegans*, in opposition to a hydrogen peroxide treatment that induced oxidative stress. The equation $F(3,236)=44.73$, $p<0.0001$ was used to run the one-way ANOVA for time to paralysis. The Tukey test demonstrated differences between the control group and all of the experimental groups. The equation $F(3,16)=4.72072$, $p=0.021252$ was used to run the one-way repeated measures ANOVA for egg-laying. The Fisher test demonstrated differences between the control group and the mechanosensory and chemosensory groups. It was concluded that the application of nonassociative learning to *C. elegans* reverses the negative effects of oxidative stress, stimulating mobility and egg-laying.

Introduction

Alzheimer's disease results in learning and memory deficits due to toxic changes in the brain. Alzheimer's patients commonly display a number of different symptoms—extreme memory loss, problem-solving handicaps, difficulty with familiar tasks, confusion with time or place, problems with vision and spatial relationships, newly developed speaking problems, object loss, poor judgement, social withdrawal, and personality changes.[3] Proteins known as amyloid-β (Aβ) accumulate in the brain and prevent neurons from firing; the result is reduced brain function as the neurons die and tissues wither.[1] A brain affected by Alzheimer's will become significantly smaller as the disease progresses.[1] Oxidative stress is also a major contributor to Alzheimer's.[2] It is caused by the overproduction of free radicals.[2] Free radicals are molecules with unpaired electrons; they are highly reactive and can negatively impact an organism. This study would further the research previously done on how cognitive processing mitigates the effects of amyloid-β and expand it to the study of the effects of oxidative stress.

Caenorhabditis elegans, a species of nematode, is one model organism that has been used to study Alzheimer's disease. *C. elegans* is useful for cognitive processing research for a number of reasons. First, its short lifespan allows one to conduct a large number of trials in a short period of time.[4] Second, its simple genetic code makes it easy to modify and isolate particular genes.[4] Lastly, the well-defined and easily accessible nervous system allows for easy modification and quantification of specific proteins, such as amyloid-β, oxidative stress, and their by-products.[4] This characteristic makes it relatively straightforward to quantify the dependent variable in studies related to Alzheimer's disease.

Some postulated causes of Alzheimer's have included advanced glycation endproducts, excess free radicals, insulin signaling, autophagy, amyloid-β deposits, and homologues of related genes, such as apolipoprotein E-e4.[2] Reducing the amounts of these substances, especially the free radicals created by oxidative stress, is an option that has been studied.[2] Associative and nonassociative learning, both types of cognitive processing, have been studied using *C. elegans*.[5] Associative learning is where the relationship between two stimuli is learned and includes taste, smell, temperature, and oxygen as conditioned stimuli.[5] Nonassociative learning occurs when behavior toward a stimulus changes without any apparent associated stimulus and includes mechanosensory and chemosensory habituation.[5] Mechanosensory habituation involves stimulation of the nematodes' touch organs.[5] Chemosensory habituation involves exposing the nematodes to chemical stimuli and observing their response–movement towards the source of the odor or an abrupt change in direction away from the source of the odor.[5] *C. elegans* demonstrates extreme sensitivity to cognitive processing, rendering it well-suited for experiments involving learning and memory.[5]

There are three main subcategories of nonassociative learning: mechanosensory habituation, chemosensory habituation, and habituation to a novel environment. For the mechanosensory habituation each test subject is touched with an inoculation loop at five second intervals. Chemosensory habituation incorporated an odorant known as diacetyl, an attractive odorant for *C. elegans*. Habituation to a novel environment involved introduction to a new plate and exploration of that plate.

Oxidative stress is caused by an organism's inability to counteract the production of free radicals. Oxidative stress can be induced in *C. elegans* using a hydrogen peroxide (H_2O_2) treatment. Exposure of *C. elegans* to oxidative stress results in paralysis and can be measured using common locomotion methods, in this case prepulse inhibition (PPI). PPI is performed by delivering acoustic, tactile, or light stimuli and recording movement changes in the test subjects. PPI can be used to measure oxidative stress, which is demonstrated through paralysis in *C. elegans*. Oxidative stress can also cause inhibition of egg-laying and growth rate, resulting in additional possibilities for measuring the progression of oxidative stress. Impending paralysis would be represented as a sensorimotor gating deficit in the test subjects. Sensorimotor gating deficit is the inability to filter out a prestimulus to focus on a subsequent stronger stimulus.

Previous research by the author in this field focused on how nonassociative cognitive processing affects amyloid-β production in *C. elegans*. Mechanosensory habituation, chemosensory habituation, and habituation to a novel environment were used as the different experimental groups for the independent variable. As previously stated, amyloid-β accumulates in plaques in the brain that prevent neurons from firing and cause tissue decay. When the amyloid-β plaques progress to the muscular system in *C. elegans*, paralysis results. The worms in the test groups, which were subjected to one of the three types of habituation, took longer to become paralyzed. Less worms became paralyzed in the test groups than in the control group. The equation $F(4,147)=16.9544$, $p<0.00001$ was used to run the one-way analysis of variance (ANOVA) test, which demonstrated a significant difference between the test groups. Using a Scheffé test, it was found that a difference existed between the control group and the test groups, suggesting that the use of nonassociative learning had a positive effect in mitigating the buildup of Aβ in *C. elegans*. This research expands the previous study by exploring the impact of nonassociative learning on oxidative stress.

Ardiel and Rankin (2010) studied the development of short and long term memory in *C. elegans* using associative learning and two types of nonassociative learning, mechanosensory and chemosensory habituation.[5] Morcos and Hutter (2009) experimented with a variety of different nervous system products and processes, including advanced glycation endproducts, oxidative stress, insulin signaling, autophagy, homologues of related genes, and amyloid-β. All were found to contribute significantly to the aging process of *C. elegans* and the progression of Alzheimer's disease in *C. elegans*. Amyloid-β was found to be a significant and measurable contributor.

Kumsta, Thamsen, and Jakob (2011) studied how oxidative stress treatment of *C. elegans* affects daily behavior.[6] Oxidative stress was stimulated in *C. elegans* by washing the worms in a rotating roller drum with hydrogen peroxide. Observations included a severe decline in egg production, reduced pharyngeal pumping, a decrease in body length, and a reduced growth rate. OxICAT, a quantitative thiol trapping technology, was used in this study to determine the oxidation status of hundreds of cellular proteins.

Yamazoe-Umemoto, Fujita, Iino, Iwasaki, and Kimura (2015) reported that neuromodulators, such as amyloid-β, a neuropeptide, have been linked to learning in animals.[7] Various neuromodulators are stimulated and inhibited by chemosensory habituation in this study. The movement of *C. elegans* caused by chemosensory habituation was observed and classified. This movement can be split into two categories: runs, which are straight migrations, and pirouettes, which are series of reverses and turns. The addition of an undesirable stimulus in the environment causes the worms to change migratory directions more frequently.

Alzheimer's disease is an increasingly prevalent condition in the United States. One in every ten Americans over age 65 has been diagnosed with Alzheimer's. Cognitive processing is commonly researched in conjunction with Alzheimer's because cognitive processing promotes neural growth and learning, while Alzheimer's inhibits it. Cognitive processing could potentially be utilized to mitigate the effects of Alzheimer's before it requires treatment and may also have applications for treatment. The previous study showed that nonassociative learning helps to prevent and mitigate the production of amyloid-β plaques, an Alzheimer's disease factor, and could be the subject of further studies as a way to delay the progression of the disease. The goal of this research extension was to test how nonassociative learning impacts oxidative stress.

In the proof of concept, it was hypothesized that oxidative stress would be produced in *C. elegans* by H_2O_2 treatment, resulting in increased paralysis and decreased egg-laying. In the subsequent study, it was hypothesized that the effects of oxidative stress on *C. elegans* would be reduced through the application of nonassociative learning, resulting in decreased paralysis and increased egg-laying.

Escherichia coli strain OP50 was used as a feeder host for the *Caenorhabditis elegans* cultures. The proof of concept tested whether the hydrogen peroxide treatment induced oxidative stress in *C. elegans*. A control group and a test group were used. The test group was exposed to the hydrogen peroxide treatment. The level of oxidative stress was observed using a mechanical version of prepulse inhibition (PPI) utilizing a strong stimulus and a weaker prestimulus. It was quantified using time to paralysis and egg-laying. Once the proof of concept was completed, the study itself tested how nonassociative learning could alleviate the effects of oxidative stress in *C. elegans*. There were three experimental groups–mechanosensory, chemosensory, and novel environment habituation–and a control group. The same hydrogen peroxide treatment method was utilized for all groups. The mechanosensory group underwent touch cell stimulation. The chemosensory group was exposed to an olfactory stimulus. The novel environment group was moved from one plate to another and back to the original plate. The same methods were used for quantification of oxidative stress. All lab equipment was sterilized after use and all cultures were bleached after experimentation was completed. Goggles and nitrile gloves were worn while working with the hydrogen peroxide.

Methods

The proof of concept was performed first. On the first test day, the *E. coli* plate culture was used to subculture 10 agar slant tubes. Nutrient agar was poured into the culture tubes, and the tubes were labeled. The inoculation loop was flamed. The *E. coli* Petri plate lid was opened using the clamshell technique, and one colony was picked up with the inoculation loop. The test tube mouth was flamed, and the colony was streaked onto the first slant culture (Appendix C, Image I). The test tube mouth and inoculation loop were flamed again, and the tube was capped. These steps were repeated for the rest of the tubes. All culture tubes were incubated for 24 hours at 37°C. The agar plates were poured on the same day that the slant cultures were made. The nematode growth agar was softened using a hot water bath for 30 minutes. The agar was allowed to cool to room temperature (25°C). The agar bottle mouth was flamed, and the agar was then poured into 14 Petri plates, which were labeled. The agar in the plates solidified overnight. The empty agar bottle was disposed of in the sharps bin.

The next day, the *E. coli* slant cultures were used to seed 4 of the Petri plates with *E. coli* as the food source of *C. elegans*. The first tube mouth was flamed and then *E. coli* was streaked onto the first plate. The tube mouth was flamed, and the tube was closed. The culture was spread by streaking and turning the plate counterclockwise continuously. These steps were repeated for the rest of the culture tubes and the plates. The plates were incubated overnight at 37°C. Then, *C. elegans* chunks from one culture were transferred to each plate to allow the *C. elegans* to emerge from

induced starvation (Appendix C, Image II). The spatula was first dipped in 91% isopropyl alcohol and the alcohol was burned off. A 1 $cm.^3$ piece of agar was chunked from original culture 1 to subculture 1. These steps were repeated for subcultures 2-4. Then, the steps were repeated for all of the plates with original culture 2. Each subculture had two agar chunks on it, one from each original culture. Each plate was observed through the stereoscope. Plates 1 and 2 contained the control group, and plates 3 and 4 contained the experimental group.

The hydrogen peroxide treatment was performed next. The worms from plates 3 and 4 were collected by centrifugation in distilled water and then washed. Distilled water was used to collect all of the worms off of the plate. The centrifuge then separated out all of the worms from the water, and the water was poured off of the top. All 60 worms were incubated in 2 mL. of distilled water with 10 m*M* of H_2O_2 in a test tube at 25°C for 30 minutes. The worms were collected by centrifugation and washed with distilled water (Appendix C, Image III). The worms were cultured on the same Petri plates. The worms on plates 1 and 2 did not go through the hydrogen peroxide treatment.

The level of oxidative stress was then observed qualitatively through sensorimotor gating using prepulse inhibition (PPI). The *C. elegans* plates were placed one at a time on the stereoscope stage. The test subjects underwent 5 minutes of stimulus to establish a baseline condition. During this period the startle stimulus (tapping the plate on the counter) was presented 30 times. After five minutes, the prepulse inhibition test was conducted. The startle burst was preceded by a prepulse stimulus (tapping on the plate with a finger). The test lasted for 5 minutes with the prepulse stimuli presented at intervals of 15 s. The test was followed by a 2 minute resting period before the plate was removed from the stereoscope stage. The level of response on each plate to the startle and prepulse stimuli during the trials was recorded. A startle response can be defined as a sudden movement or sharp change of direction observed in *C. elegans* after a stimulus. Two types of data were collected: 1) the time it took each test subject to become paralyzed and 2) the number of eggs laid on the plate each day.

The experimental trials were conducted after the proof of concept was finished. The same steps were repeated for seeding the plates with *C. elegans* and conducting the hydrogen peroxide treatment. All of the plates underwent the hydrogen peroxide treatment. Once the *C. elegans* were re-seeded on the culture plates, the cognitive processing was conducted. Ten cultures were used; cultures 1-8 contained 60 worms each, while cultures 9-10 contained none (Appendix C, Image IV). Each test group was kept separate. Plates 1 and 2 were controls, plates 3 and 4 were mechanosensory habituation, plates 5 and 6 were chemosensory habituation, and plates 7 and 8 were habituation to a novel environment. Plates 9 and 10 were used as the new environment for the habituation to a novel environment group. The mechanosensory habituation group underwent touch cell stimulation. The inoculation loop was sterilized. Then, several different worms at 5 different spots on the Petri plate were touched with the loop at 5-second intervals. The loop was flamed in between spots. The spots where worms were touched were marked on the bottom of the Petri plate. The chemosensory habituation group underwent repeated exposure to an attractive odorant (diacetyl). One drop of diacetyl was pipetted onto a region that was clear of both *E. coli* and *C. elegans*. The location of the drop was marked on the bottom of the Petri plate. The habituation to a novel environment group was introduced to a new environment (a different Petri plate). 60 worms were moved from plate 7 to plate 9 and from plate 8 to plate 10 with a toothpick. The toothpick was changed after every 5 worms. The worms were allowed to explore the plate for 5 minutes. The worms were moved back to their original plates. This process was repeated 24 hours later for the second stage of the habituation (Appendix A, Figure III). The control group did not go through any type of cognitive processing. The same methods were repeated for the prepulse inhibition trial and the data quantification. All plates and slant cultures were bleached and disposed of after experimentation was over.

Numerous safety precautions were taken during the experimentation. Goggles were worn while the Bunsen burner was in use, and all instruments were sterilized. The toothpicks used to transfer the worms were all bleached. The inoculation loop, the spatula, and the tube mouths were all sterilized using the Bunsen burner. The plates were bleached after use, and the lab bench was wiped down (sterilized) after each test day. All safety guidelines for the use of hydrogen peroxide were followed.

Results

To quantify the level of oxidative stress in *C. elegans*, the number of days to paralysis for each test subject and the number of eggs laid per plate were measured. Since there were two plates per group, the values for each group were averaged between the two plates. In the proof of concept, the control group took longer to become paralyzed and laid more eggs than the group that was exposed to the hydrogen peroxide. In the experimental trial, the control group took less time to become paralyzed and laid less eggs than the groups that were exposed to the nonassociative learning. Prepulse inhibition was also used to qualitatively evaluate *C. elegans*. In the proof of concept, the experimental subjects exhibited extreme movement inhibition as compared to the control subjects. The control subjects exhibited sensorimotor gating and only responded to the stimulus. The experimental subjects showed little sensorimotor gating and responded to both the pre-stimulus and the stimulus. The “pre-stimulus” is the weak prepulse stimulus (tapping the plate with a finger), while the “stimulus” is the strong stimulus (tapping the plate on the counter). In the experimental trial, the experimental subjects displayed a higher level of sensorimotor gating as compared to the control subjects. The control subjects exhibited little to no sensorimotor gating and responded to both the pre-stimulus and the stimulus. The mechanosensory and novel environment groups exhibited a high level of sensorimotor gating. The chemosensory group exhibited moderate sensorimotor gating; some of the subjects responded to both stimuli and some did not. The sensorimotor gating level increased significantly when comparing the prepulse inhibition exhibited before and after habituation.

In the proof of concept, the control group took longer to become paralyzed than the experimental group did. The experimental group was exposed to the hydrogen peroxide treatment, while the control group was not. Both groups exhibited the same range (3.0000). However, the control group exhibited higher mean and median numbers of days as compared to the experimental group (Appendix B, Table I).

In the proof of concept, the control group laid more eggs than the experimental group. The control range was 408.5, while the experimental range was 0.5. The control group also exhibited higher mean and median values as compared to the experimental group (Appendix B, Table II).

In the experimental trial, the control group became paralyzed in a shorter time period when compared to the experimental groups. The control group

had the smallest mean and median. The mechanosensory group had third highest mean, the chemosensory group had the highest mean, and the novel environment group had the second highest mean. The experimental groups all had the same median, which was higher than the control median (Appendix B, Table III).

In the experimental trial, the control group laid significantly less eggs than the experimental groups. The control group had the smallest mean and median. The mechanosensory group had highest mean and median, the chemosensory group had the second highest mean and median, and the novel environment group had the second lowest mean and median. The experimental groups all displayed a greater range than the control group (Appendix B, Table IV).

In the proof of concept, the control group took longer overall to become paralyzed than the experimental group, which received the hydrogen peroxide treatment. The control group's data is concentrated on day 8, while the experimental group maintains a more even distribution (Appendix B, Figure IV).

In the proof of concept, the control group laid more eggs than the experimental group over a period of 4 days. The control group laid hundreds of eggs, compared to the experiment group's total of less than 20 eggs. The control group also showed a greater change in egg-laying over time (Appendix B, Figure V).

In the experimental trial, the control group showed an even distribution of paralysis over a period of 6 days with a concentration on day 9. All of the experimental groups showed a heavy concentration of paralysis on day 14 (Appendix B, Figure VI).

In the experimental trial, the control group laid less than 20 eggs over a period of 6 days. The mechanosensory and chemosensory groups laid over 100 eggs each, while the novel environment group laid around 50 eggs. The mechanosensory and chemosensory groups demonstrated the most change over time (Appendix B, Figure VII).

The time to paralysis data for the proof of concept was analyzed using an independent samples t-test with an alpha value of 0.05. The t-test was run with H_0: $\mu1=\mu2$ and H_1: $\mu1\neq\mu2$. The equation $T(42)=3.68$, $p=0.0007$ was also used to run the t-test. An T value of 3.68 was reported, which suggests that there was a significant difference between the test groups. A P-value of 0.0007 was also reported, indicating that there is a 0.07% chance that the results were due to random chance alone (Appendix B, Table V).

The egg-laying data for the proof of concept was analyzed using an one-way repeated measures analysis of variance (ANOVA) test with an alpha value of 0.05. The ANOVA was run with H_0: $\mu1=\mu2$ and H_1: $\mu1\neq\mu2$. The equation $F(1,8)=3.42058$, $p=0.138072$ was also used to run the ANOVA. An F value of 3.42058 was reported, which suggests that there was not a significant difference between the test groups. A P-value of 0.138072 was also reported, indicating that there is a 13.8072% chance that the results were due to random chance alone (Appendix B, Table VI).

The time to paralysis data for the experimental trial was analyzed using a one-way ANOVA test with an alpha value of 0.05. The ANOVA was run with H_0: $\mu1=\mu2=\mu3=\mu4$ and H_1: at least one mean is different from the others. The equation $F(3,236)=44.73$, $p<0.0001$ was also used to run the ANOVA. An F value of 44.73 was reported, which suggests that there was a significant difference between the test groups. A P-value of less than 0.0001 was also reported, indicating that there is a 0.01% chance that the results were due to random chance alone (Appendix B, Table VII).

The differences between the groups were located using a Tukey test with an alpha value of 0.05. A significant difference was found between the control group and each of the experimental groups (Appendix B, Table VIII).

The egg-laying data for the experimental trial was analyzed using an one-way repeated measures ANOVA test with an alpha value of 0.05. The ANOVA was run with H_0: $\mu1=\mu2=\mu3=\mu4$ and H_1: at least one mean is different from the others. The equation $F(3,16)=4.72072$, $p=0.021252$ was also used to run the ANOVA. An F value of 4.72072 was reported, which suggests that there was a significant difference between the test groups. A P-value of 0.021252 was also reported, indicating that there is a 2.1252% chance that the results were due to random chance alone (Appendix B, Table IX).

The differences between the groups were located using a Fisher least significant difference (LSD) test with an alpha value of 0.05. A significant difference was found between the control group and both the mechanosensory and chemosensory groups (Appendix B, Table X).

Discussion

The purpose of this study was to extend previous research on the impact of cognitive processing on the development of amyloid-β plaques in *C. elegans* to exploring the impact of nonassociative learning on oxidative stress. Both amyloid-β plaques and oxidative stress are major factors in the development of Alzheimer's disease. One in ten Americans over age 65 has been diagnosed with Alzheimer's. The goal of the this research was to determine whether nonassociative learning should be further studied as either a treatment option or a preventative measure for Alzheimer's disease.

The hypothesis was partially supported in the proof of concept. The t-test for the proof of concept time to paralysis data showed that the hydrogen peroxide treatment did result in a significant difference between the groups, but the repeated measures ANOVA for the egg-laying did not yield statistically significant results. It was concluded that the hydrogen peroxide treatment did produce oxidative stress in *C. elegans*. In the experimental trial, it was hypothesized that the effects of oxidative stress would be reduced by the application of nonassociative learning. This hypothesis was supported. The one-way ANOVA for the experimental trial time to paralysis data showed a significant difference between the groups. The post-hoc Tukey test demonstrated that the differences lay between the control and the mechanosensory, chemosensory, and novel environment groups. The repeated measures ANOVA for the egg-laying also showed a significant difference between the groups. The post-hoc Fisher LSD test displayed that the differences lay between the control and mechanosensory groups and between the control and chemosensory groups. It was concluded that all three types of nonassociative learning mitigated the effects of oxidative stress in *C. elegans*.

The results of this study are consistent with those of previous research. Kumsta, Thamsen, and Jakob (2011) also found that hydrogen peroxide treatment produces oxidative stress in *C. elegans*. Yamazoe-Umemoto, Fujita, Iino, Iwasaki, and Kimura (2015) found that both neuropeptide and dopamine signalings are necessary for a response to odor learning. This study confirmed that nonassociative learning cognitive processing can stimulate neuropeptides, such as the amyloid precursor protein, to the point that the effects of oxidative stress are mitigated.

It can be concluded that hydrogen peroxide treatment produces oxidative stress in *C. elegans*. It can also be concluded that the use of nonassociative learning mitigates the effects of oxidative stress by increasing egg-laying and locomotion. There are a few sources of error in this study. If any of the Petri plates had become contaminated, the contamination could have affected the growth of both *E. coli* and *C. elegans* and skewed the data. Another source of error is the manual quantification of paralysis and egg-laying; the number of paralyzed worms could have been miscounted on any test day. This development would cause the data for some groups to be an inaccurate representation of the population. Additionally, only two Petri plates were used for each group, which does not constitute a very large sample size.

If research were to be continued, oxidative stress could be measured using reactive oxygen species, resulting damage to biomolecules, or antioxidant levels. Any of these methods would allow for a more direct quantification of the progression of oxidative stress in *C. elegans*. More Petri plates could also be used for each group. The use of at least 10 plates for each type of habituation would provide a minimum of 300 worms from which to obtain data and would minimize the possibility that the results were related to random chance or a mutation in the genome or environment.

In an extension to this research, one could study different types of cognitive processing, such as associative learning, which is connected to the use of learned stimuli. Different factors relating to Alzheimer's disease could also be used. The gene apolipoprotein E-e4 has been found to be the risk gene with the greatest influence on Alzheimer's disease. Sequencing each test subject and identifying risk genes would allow for studying the effect of cognitive processing on genetic factors. Additionally, the effect of the surrounding environment and diet on the development of Alzheimer's could be studied.

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APPENDIX A

Figure I: Proof of concept experimental design diagram

<table>
<tr><td colspan="2">Title of the Experiment:
The effect of nonassociative learning on oxidative stress in Caenorhabditis elegans: A potential application for Alzheimer's disease research</td></tr>
<tr><td colspan="2">Hypothesis:
H_2O_2 treatment produces oxidative stress in C. elegans, resulting in increased paralysis and decreased egg-laying.</td></tr>
<tr><td colspan="2">Independent Variable:
presence of H_2O_2 treatment</td></tr>
<tr><td>no H_2O_2</td><td>10 mM of H_2O_2</td></tr>
<tr><td>30 trials</td><td>30 trials</td></tr>
<tr><td colspan="2">Dependent Variable:
oxidative stress (time to paralysis and egg-laying)</td></tr>
<tr><td colspan="2">Constants:
species of model organism (C. elegans), living conditions of nematodes, strain of E. coli (OP50), amount of nematode growth agar, H_2O_2 treatment methods</td></tr>
<tr><td colspan="2">Control:
nematode group without H_2O_2 treatment applied</td></tr>
</table>

Figure II: Study experimental design diagram

<table>
<tr><td colspan="3">Title of the Experiment:
The effect of nonassociative learning on oxidative stress in Caenorhabditis elegans: A potential application for Alzheimer's disease research</td></tr>
<tr><td colspan="3">Hypothesis:
The application of nonassociative learning reduces the effects of oxidative stress on C. elegans and results in decreased paralysis and increased egg-laying.</td></tr>
<tr><td colspan="3">Independent Variable:
type of cognitive processing (nonassociative learning)</td></tr>
<tr><td>mechanosensory habituation</td><td>chemosensory habituation</td><td>habituation to a novel environment</td></tr>
<tr><td>60 trials</td><td>60 trials</td><td>60 trials</td></tr>
<tr><td colspan="3">Dependent Variable:
oxidative stress (time to paralysis and egg-laying)</td></tr>
<tr><td colspan="3">Constants:
species of model organism (C. elegans), living conditions of nematodes, strain of E. coli (OP50), amount of nematode growth agar, H_2O_2 treatment methods</td></tr>
<tr><td colspan="3">Control:
nematode group without any cognitive processes applied</td></tr>
</table>

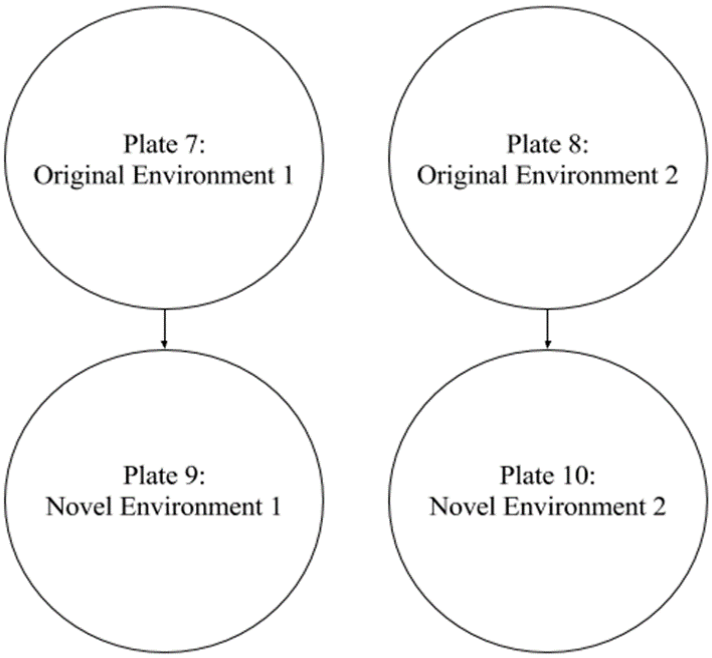

Figure III: Diagram of methods for habituation to a novel environment

APPENDIX B

Table I: Descriptive Statistics for Time to Paralysis, Proof of Concept

Statistics

Variable	POC Group 1	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
POC Days to Paralysis	1	30	0	7.8333	0.1081	0.5921	5.0000	8.0000	8.0000	8.0000	8.0000
	2	30	0	6.9333	0.2194	1.2015	5.0000	6.0000	7.0000	8.0000	8.0000

Table I displays the descriptive statistics for the number of days to paralysis for each test subject in the control and experimental proof of concept groups. The control group (1) took an average of 7.83 days to become paralyzed, while the experimental group (2) took an average of 6.93 days. The control group was not exposed to the hydrogen peroxide treatment, while the experimental group was.

Table II: Descriptive Statistics for Eggs Laid, Proof of Concept

Statistics

Variable	POC Group 2	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
POC Eggs Laid	1	3	0	315.3	120.5	208.6	86.5	86.5	364.5	495.0	495.0
	2	3	0	4.8333	0.1667	0.2887	4.5000	4.5000	5.0000	5.0000	5.0000

Table II displays the descriptive statistics for the number of eggs laid for each proof of concept group. The control group (1) laid an average of 315.30 eggs, while the experimental group (2) laid an average of 4.83 eggs.

Table III: Descriptive Statistics for Time to Paralysis, Experimental Trial

Statistics

Variable	ET Group 1	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
ET Days to Paralysis	1	60	0	10.8000	0.2502	1.9380	9.0000	9.0000	10.0000	13.0000	14.0000
	2	60	0	13.3833	0.1947	1.5081	9.0000	14.0000	14.0000	14.0000	14.0000
	3	60	0	13.6167	0.1596	1.2363	9.0000	14.0000	14.0000	14.0000	14.0000
	4	60	0	13.4000	0.1844	1.4285	9.0000	14.0000	14.0000	14.0000	14.0000

Table III displays the descriptive statistics for the number of days to paralysis for each test subject in the experimental trial groups. The control group (1) took an average of 10.8000 days. The mechanosensory group (2) took an average of 13.3833 days. The chemosensory group (3) took an average of 13.5167 days. The novel environment group (4) took an average of 13.4000 days.

Table IV: Descriptive Statistics for Eggs Laid, Experimental Trial

Statistics

Variable	ET Group 2	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
ET Eggs Laid	1	5	0	0.4000	0.2915	0.6519	0.0000	0.0000	0.0000	1.0000	1.5000
	2	5	0	61.80	26.41	59.06	17.50	19.00	32.00	119.50	156.50
	3	5	0	61.40	22.93	51.27	13.00	13.25	50.50	115.00	117.50
	4	5	0	14.300	1.488	3.328	10.000	11.000	15.500	17.000	18.500

Table IV displays the descriptive statistics for the number of eggs laid for each experimental trial group. The control group (1) laid an average of 0.40 eggs. The mechanosensory group (2) laid an average of 61.80 eggs. The chemosensory group (3) laid an average of 61.40 eggs. The novel environment group (4) laid an average of 14.30 eggs.

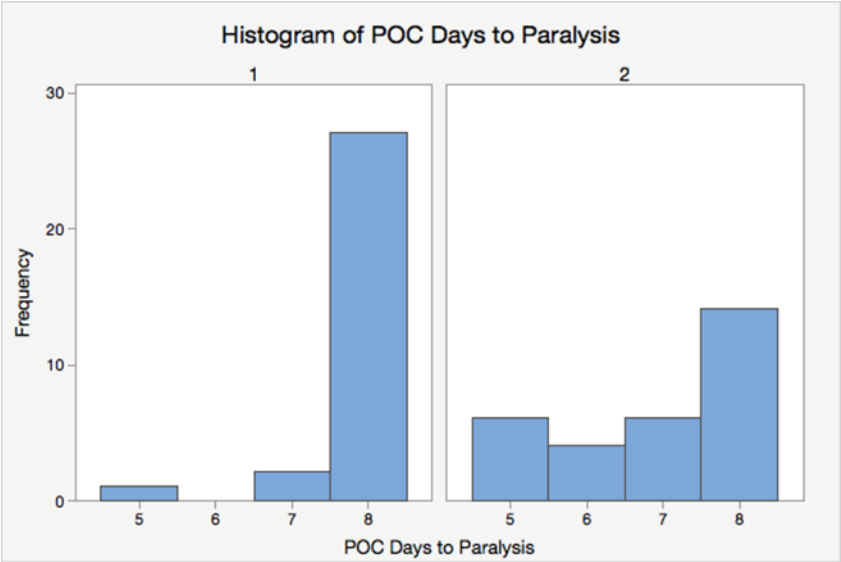

Figure IV: Histogram for time to paralysis, proof of concept. Figure displays the histograms for the number of days to paralysis for each test subject in the proof of concept groups. The control group (1) mostly became paralyzed at 8 days, while the experimental group (2) displayed a more even spread over 5-8 days.

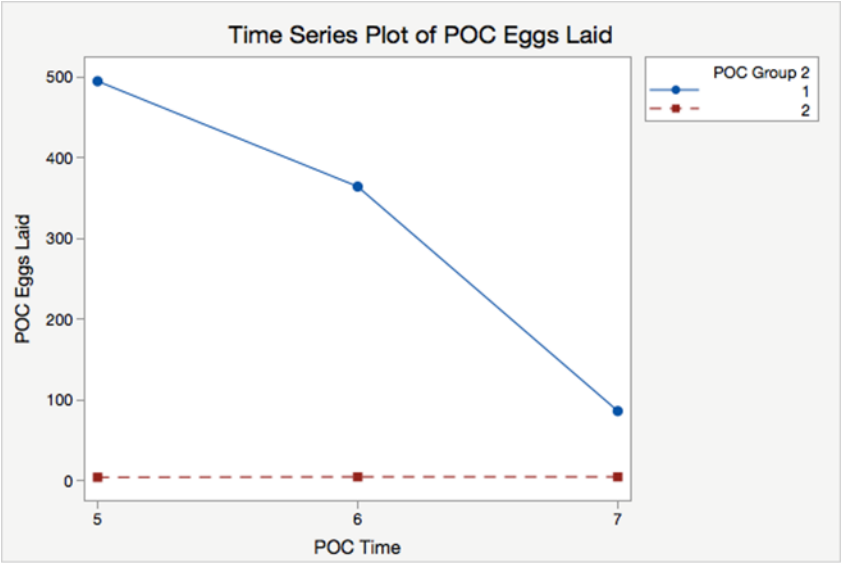

Figure V: time series plot of eggs laid, proof of concept. Figure displays the time series plot for the number of eggs laid for each proof of concept group. The control group (1) laid significantly more eggs than the experimental group (2). The control group also demonstrates a downward trend in egg-laying over time. The experimental group stays relatively constant.

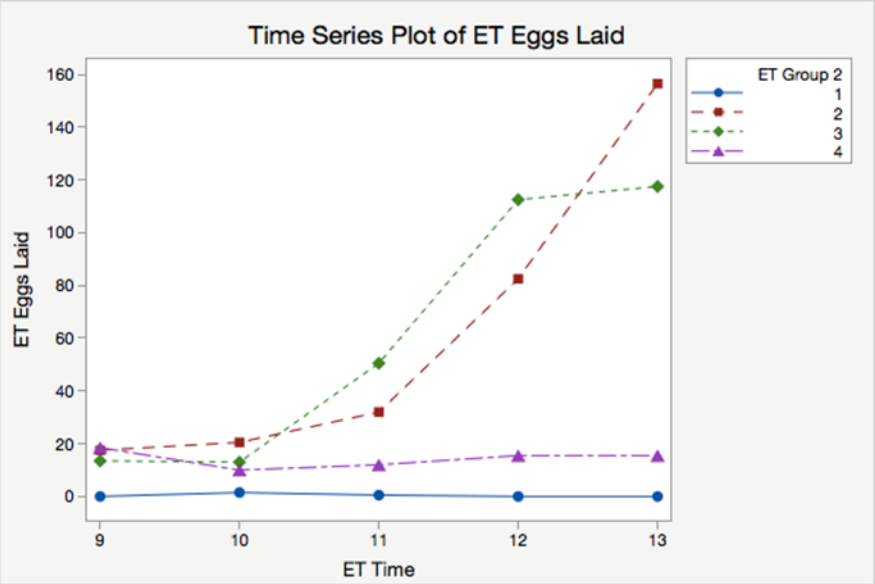

Figure VII: time series plot of eggs laid, experimental trial. Figure displays the time series plot for the number of eggs laid for each proof of concept group. The control group (1) and the novel environment group (4) demonstrate a similar constant trend, though the novel environment group has an overall higher number of eggs. Both of the other experimental groups (2-3) show a general upward trend.

Table V: Independent T-Test for Proof of Concept Time to Paralysis Data (α=0.05)

T-value	degree of freedom
3.68	42

P-value: 0.0007

Table V displays the summary table for the independent samples t-test run for the days to paralysis data for the proof of concept. It can be seen that the hydrogen peroxide treatment had a statistically significant effect on the amount of time it took each subject to become paralyzed at α=0.05, T(42)=3.68, p=0.0007.

Table VI: Repeated Measures ANOVA For Proof of Concept Egg-Laying Data (α=0.05)

source	sum of squares	degree of	mean square	F
between	86769.225	1	86769.225	3.42058
within	206412.5	8	25801.5624	
total	101467.15	4	25366.7875	

P-value: 0.138072

Table VI displays the summary table for the one-way repeated measures ANOVA run for the egg-laying data for the proof of concept. It can be seen that the hydrogen peroxide treatment did not have a statistically significant effect on the number of eggs laid per group over a period of three days at α=0.05, F(1,8)=3.42058, p=0.138072.

Table VII: One-Way ANOVA For Experimental Time to Paralysis Data (α=0.05)

source	sum of squares	degree of	mean square	F
between	322.033	3	107.344	44.73
within	566.367	236	2.400	
total	888.400	239		

P-value: <0.0001

Table VII displays the summary table for the one-way ANOVA run for the days to paralysis data for the experimental trial. It can be seen that the nonassociative learning had a statistically significant effect on the amount of time it took each subject to become paralyzed at α=0.05, F (3,236)=44.73, p<0.0001.

Table VIII: Tukey Test For Experimental Time to Paralysis Data (α=0.05)

difference of levels	difference of	SE of	95% Cl	T-value	adjusted P
2-1	2.5833	0.2828	(1.8514,	9.13	<0.0001
3-1	2.8167	0.2828	(2.0847,	9.96	<0.0001
4-1	2.6000	0.2828	(1.8680,	9.19	<0.0001
3-2	0.2333	0.2828	(-0.4986,	0.82	0.8426
4-2	0.0167	0.2828	(-0.7153,	0.06	0.9999
4-3	-0.2167	0.2828	(-0.9486,	-0.77	0.8697

individual confidence level=98.97%

Table VIII displays the summary table for the Tukey test run for the days to paralysis data for the experimental trial. A significant difference was found between groups 1 and 2, 1 and 3, and 1 and 4.

Table IX: Repeated Measures ANOVA For Experimental Egg-Laying Data (α=0.05)

source	sum of squares	degree of	mean square	F
between	15198.7375	3	5066.2456	4.72072
within	24513	16	1532.0625	
total	12878.325	12	1073.1938	

P-value: 0.021252

Table IX displays the summary table for the one-way repeated measures ANOVA run for the egg-laying data for the experimental trial. It can be seen that the nonassociative learning had a statistically significant effect on the number of eggs laid per group over a period of 5 days at α=0.05, F(3,16)=4.72072, p=0.021252.

Table X: Post Hoc Fisher Least Significant Difference Test for Experimental Egg-Laying Data (α=0.05)

Fisher Individual Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
2-1	61.40	24.76	(8.92, 113.88)	2.48	0.0246
3-1	61.00	24.76	(8.52, 113.48)	2.46	0.0254
4-1	13.90	24.76	(-38.58, 66.38)	0.56	0.5822
3-2	-0.40	24.76	(-52.88, 52.08)	-0.02	0.9873
4-2	-47.50	24.76	(-99.98, 4.98)	-1.92	0.0730
4-3	-47.10	24.76	(-99.58, 5.38)	-1.90	0.0752

Simultaneous confidence level = 81.11%

Table X displays the summary table for the Fisher least significant difference test run for the egg-laying data for the experimental trial. A significant difference was found between groups 1 and 2 (control and mechanosensory) and groups 1 and 3 (control and chemosensory).

Appendix C

Image I: Lab Setup for *E. coli* Agar Slant Cultures

Image II: Lab Setup for *C. elegans* Culturing

Image III: Lab Setup for Hydrogen Peroxide Centrifugation

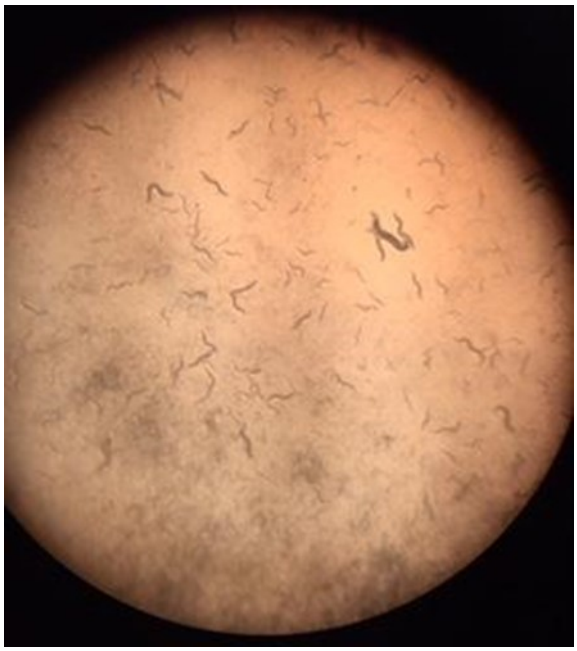

Image IV: *C. elegans* Test Plate