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Behavioral responses to gold nanoparticle exposure and H_2O_2-induced oxidative stress in *Caenorhabditis elegans*

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ABSTRACT Gold nanoparticles (AuNPs) have been utilized in many biomedical disciplines, most notably cancer therapy and drug delivery. Recent research suggests that with specific peptide manipulation, AuNPs can deliver drugs across the blood-brain barrier (BBB), allowing for treatment of neurodegeneration and other neurological afflictions. Neurodegeneration has been shown to be caused by oxidative stress. The present experiment aimed to assess the effects of AuNPs on *C. elegans* behavior that had undergone H_2O_2-induced oxidative stress. It was predicted that worms exposed to both H_2O_2 and AuNPs would have higher survival, mechanosensation, and thrashing rates than worms only exposed to H_2O_2. After worms were exposed to H_2O_2 solution and AuNP solution, mortality, mechanosensation, and thrashing data were obtained. Nematodes who were exposed to both AuNPs and H_2O_2 ($\mu = 51.1$ thrashes/min) did not experience the same decrease in locomotion as the worms only exposed to H_2O_2 ($\mu = 30.8$ thrashes/min), $p = 0.05$. This suggests that AuNP exposure may reverse the negative effects of H_2O_2 on *C. elegans* movement. Worms exposed to AuNPs by liquid vehicle in the first experiment exhibited significantly less locomotion ($\mu = 46.3$ thrashes/min) than worms exposed to AuNPs through their food in the second experiment ($\mu = 119.6$ thrashes/min), $p < 0.001$. Results from this study assert that AuNPs may help relieve symptoms of oxidative stress and neurodegeneration, especially in neuromuscular activity in *C. elegans*.

INTRODUCTION

Introduced as a model organism by Dr. Sydney Brenner, Ph.D. in the mid- to late twentieth century, *Caenorhabditis elegans* has been readily studied in numerous scientific fields, as the free-living, microscopic nematode allows for relative simplicity in its culture, maintenance, observation, and analysis.[14] Two of the fields that often utilize *C. elegans* are behavioral neuroscience and nanotechnology; however,

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these fields are rarely studied in *C. elegans* in tandem.

One previous study found that exposure to certain nanomaterials, specifically silica nanoparticles and silver nanoparticles, can have a negative impact on *C. elegans* motility and behavior.[19] Silver nanoparticles have also been found to induce multigenerational toxicity in *C. elegans*.[13] Gold nanoparticles (AuNPs) specifically have been shown to negatively affect *C. elegans* reproduction, and in cases of high AuNP concentration, they have been shown to have a toxic effect on *C. elegans*.[23] However, the behavioral effect of AuNPs on *C. elegans* has not been studied at length. In other organisms, AuNPs have been shown to play a role in the regeneration of neurons,[15] suggesting that AuNPs may have positive implications in future neurological and behavioral research.

While research on the effect of AuNPs on *Caenorhabditis elegans* behavior is scarce, many previous studies provide insight into the behavioral effects of H_2O_2, a reactive oxygen species (ROS), on *C. elegans.* Previous literature has established H_2O_2 as an ROS known to induce oxidative stress in *C. elegans*.[12,22] ROS-induced oxidative stress in *C. elegans* is often characterized by a loss in mobility, indicating a loss of function at the neuromuscular junction (NMJ).[22]

Oxidative stress has been found to play an important role in neurodegeneration, meaning that it is vital to the development of human conditions like amyotrophic lateral sclerosis (ALS), Parkinson's, Alzheimer's, and other neurodegenerative disorders.[3,5,7,10,24] Treatment of such disorders has proved incredibly difficult due to the highly impermeable blood-brain barrier (BBB), but recent research has revealed the possibility of peptide sequence manipulation of AuNPs to cross the BBB and deliver drugs to the brain, making treatment of neurodegenerative and other neurological disorders possible.[16]

The present study aimed to discover if AuNPs themselves could have an effect on the neurodegeneration caused by H_2O_2-induced oxidative stress in *C. elegans*, considering their previous implications in neural regeneration.[15] This was investigated by assessing the effect of AuNPs and H_2O_2 on mechanosensation, thrashing frequency, and survival. It was hypothesized that AuNPs would have an effect on *C. elegans* behavior and survival due to previous literature showing that *C. elegans* are affected by exposure to metal nanoparticles.[13,15,19,23] More specifically, a positive effect was predicted, in that survival, mechanosensation, and locomotion rates would increase when compared to worms not exposed to AuNPs. This hypothesis was based upon the regenerative powers of AuNPs in previous studies.[15] It was also hypothesized that, as shown in other papers, H_2O_2 would affect *C. elegans* behavior and survival, and it was predicted that H_2O_2 exposure would decrease rates of survival, response to mechanical stimuli, and locomotion in *C. elegans*, indicating the induction of oxidative stress.[12,22] In addition, it was predicted that there would be an interaction between the AuNP and H_2O_2 exposure variables in that worms exposed to both AuNPs and H_2O_2 would not experience the same negative effects as those only treated with H_2O_2. This prediction was also based on the previously mentioned finding that AuNPs play a role in the regeneration of neurons.[15]

MATERIALS AND METHODS

Preparation of Nematode Growth Plates

The organism observed in the present experiment was *Caenorhabditis elegans*, a microscopic nematode worm cultured on Petri dishes for use in the laboratory. A starter plate of nematode growth media (NGM) seeded with *Escherichia coli* OP50 and containing N2 wild-type *C. elegans* was obtained from the Andalusian Center for Developmental Biology in Seville, Spain. After three days, the starter plate was chunked and transferred onto fresh NGM plates seeded with *E. coli* OP50 and stored at room temperature. Lysogeny broth media was prepared, inoculated with the *E. coli* OP50 from the starter plate, and incubated overnight at approximately 27ºC to ensure sufficient bacterial growth. Nematode growth media was prepared using the NGM recipe by Cold Spring Harbor Protocols,[17] and once the media was prepared, NGM plates were poured using aseptic technique,

seeded with 50 µL of liquid *E. coli* OP50 culture, and incubated overnight at 27ºC. Nematode worms were then chunked onto the new NGM plates, and a 1:1 ratio of 1 M NaOH and bleach was introduced to the plates to eliminate bacterial cross-contamination.[21] Nematode-containing plates were then stored in a temperature-controlled room set to 20ºC until further use.

Synthesis and Characterization of AuNPs

Potassium tetrachloroaurate (III) with a concentration of 1.5 mM and citrate with a concentration of 33.8 mM was obtained, and these materials were used to create AuNPs via sonication with a Misonix Sonicator 3000 set to 15 watts and an amplitude of 13% for approximately 5 minutes.[6] After creation, the AuNP solution was centrifuged,[1] and the pellet was resuspended in ultrapure water equal to its original volume. UV-visible spectroscopy was utilized to characterize the resuspended AuNP solution using a Jasco V-500 series spectrophotometer. The peak height and absorbance of the AuNP solution was recorded along with the absorbance found at 450 nm to calculate AuNP size and concentration. The diameter was found using the equation: $d = e^{(B1\frac{A_{spr}}{A_{450}} - B2)}$, and then used in the equation: $d = \frac{A_{spr}(5.89x10^{-6})^{\frac{1}{C_2}}}{C_{Au}e(C_1)}$ to find the concentration of the AuNPs.[8] The concentration of AuNPs could then be used to find the concentration of Au in the solution. The size of the AuNPs used in this experiment were 11.15 nm with the concentration of gold in solution being 0.133 g/L.

Introduction to Oxidative Stress and AuNPs

The first experiment employed a 2x2 factorial design in which the two independent variables were AuNP exposure and H_2O_2-induced oxidative stress. *C. elegans* were separated into four treatment groups, exposed to one of two concentrations of H_2O_2 solutions, and then exposed to one of two AuNP solutions in 50% K-medium (31.68 mM KCl and 51.37 mM NaCl).[23] Each of the four treatment groups were exposed to one of the following solutions of H2O2 and AuNPs: 0 mM H_2O_2 and 0 mg/L AuNP (double control); 0 mM H_2O_2 and 5.95 mg/L AuNP; 10 mM H_2O_2 and 0 mg/L AuNP; and 10 mM H_2O_2 and 5.95 mg/L AuNP (double experimental). Each group was introduced to its respective H_2O_2 solution in a 15 mL tube for 30 minutes while gently vortexing. Worms were then collected via centrifugation and introduced to their respective AuNP solution in Petri dishes for 12 hours. All worms were then transferred to 50% K-medium for an additional 12 hours.

Mortality and Behavioral Assays

Mortality was scored immediately, and worms were considered deceased if they did not respond to repeated poking with a platinum worm pick.[23] Assays were conducted on worms during the L4 and adult stages of their life cycle. These stages of the worms' life cycle were chosen to ensure reproductive and developmental maturity regardless of whether nutrients were available to them.[2,20] By limiting the ages of the worms to those already developed, it negates any behavioral differences that may have occurred due to age differences. Mechanosensation assays were conducted by stroking the end of the nematodes with the tapered edge of a sterilized eyebrow hair and recording the number of strokes needed to elicit a response from each worm.[4] Mechanosensation scores were given on a 1 - 4 scale. Worms that responded after one touch were given a score of 1, those who responded after two touches were given a score of 2, those who responded after greater than two touches were given a score of 3, and those who did not respond were given a score of 4. Thrashing assays were conducted by introducing individual worms to a drop of M9 buffer (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, and 1 mM $MgSO_4$).[18] The number of thrashes exhibited by the worms were scored over the course of 1 minute.[21]

Introduction of AuNPs through Bacterial Food Source

The second experiment in the present study consisted of introducing the nematodes to AuNPs through their *E. coli* OP50 food source. A 1:100 solution of AuNP:bacteria was prepared using 8.78 x 10^{-6} M AuNPs and an *E. coli* liquid culture with an OD600 of 0.757. NGM plates were seeded with either *E. coli* (control group) or AuNP:bacteria solution and incubated overnight

at room temperature. Worms were then chunked on to each group of NGM plates and allowed to feed for 12 hours.[11] Mortality was scored and mechanosensation and thrashing assays were conducted immediately following the 12 hours using the methods described above.

RESULTS

Mortality

Average mortality rates were computed based on mortality assays performed in the first experiment and are shown in Table 1.

Treatment	Average Mortality Rate (%)
Control (no AuNPs or H_2O_2)	23.96 ± 0.01
AuNPs only	25.92 ± 0.07
H_2O_2 only	76.52 ± 0.09
Both AuNPs and H_2O_2	69.85 ± 0.06

Table 1. Average Mortality Rates by Treatment Group.

A 2x2 between-subjects ANOVA was conducted on SPSS Statistical Software to assess the significance of the mortality rates observed in the first experiment. It was found that higher mortality rates were observed for worms exposed to H_2O_2 ($\mu = 73.18\%$, $SE = 0.04$) than for those not exposed to H_2O_2 ($\mu = 24.94\%$, $SE = 0.02$), regardless of AuNP exposure, and this finding was considered to be significantly significant, $F(1, 4) = 118.13$, $p < 0.001$. This difference can be seen in Figure 1. No significant relationship was found between AuNP exposure and mortality rate.

Mechanosensation

Mechanosensation data was scored on a scale of 1 - 4, with higher numbers indicating slower mechanosensory response times. A 2x2 between-subjects MANOVA was conducted using SPSS Statistical Software which concluded no significant relationship between AuNP exposure and mechanosensory function. However, a significant relationship was observed between exposure to oxidative stress and mechanosensation, in that worms exposed to H_2O_2 exhibited a significant delay in response times ($\mu = 2.31$, $SE = 0.25$) when compared to worms exposed to only vehicle ($\mu = 1.38$, $SE = 0.18$), $F(1, 29) = 7.16$, $p < 0.05$. These data are shown in Figure 2.

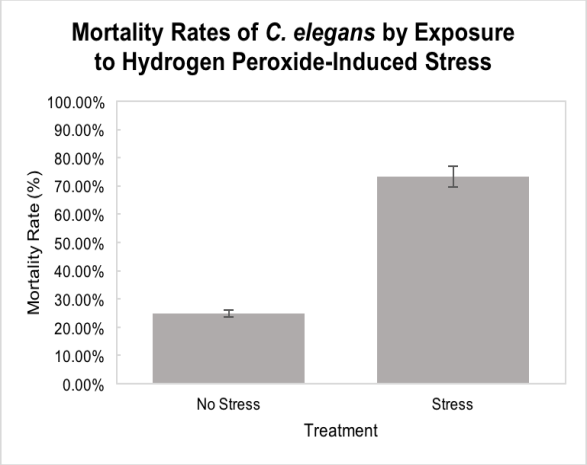

Figure 1. Average Mortality Rates of *C. elegans* Exposed to Oxidative Stress by 10 mM H_2O_2 Compared to those introduced to vehicle. Worms not exposed to H_2O_2 exhibited a 24.94 ± 0.04% average mortality rate while worms exposed to H_2O_2 exhibited an average mortality rate of 73.18 ± 0.07%. $p < 0.001$**

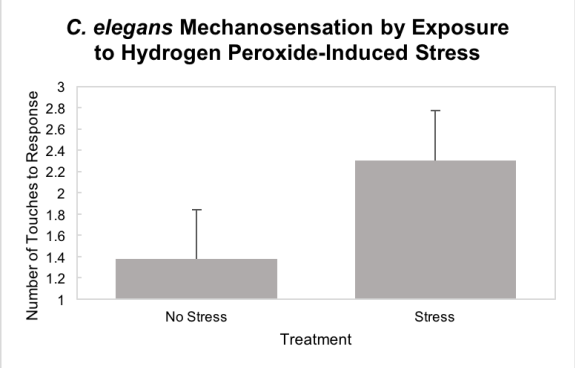

Figure 2. Average mechanosensation data for *C. elegans* exposed to oxidative stress by 10 mM H_2O_2 compared to the control. Worms that responded after 1 touch were given a score of 1, those who responded after 2 or > 2 touches were given a score of 2 or 3,

respectively, and worms who did not respond were given a score of 4. Worms not exposed to H_2O_2 responded after an average of 1.38 ± 0.73 touches while H_2O_2-treated worms responded after an average of 2.31 ± 1.05 touches. $p < 0.05$*.

Locomotion by Treatment

Average locomotion data collected from thrashing assays performed in the first experiment in this study are presented in Table 2. Values are presented in thrashes/minute.

Treatment	Thrashes/Minute
Control (no AuNPs or H_2O_2)	59.3 ± 5.1
AuNPs only	46.3 ± 18.3
H_2O_2 only	30.8 ± 20.3
Both AuNPs and H_2O_2	51.1 ± 30.3

Table 2. Average C. elegans Thrashing Data by Exposure to 10 mM H2O2 and AuNP in 50% K-medium.

A 2x2 between-subjects MANOVA was conducted to assess the significance of thrashing data obtained in the first experiment. A significant interaction was observed between the two independent variables such that worms exposed to H_2O_2 only experienced a significant decrease in thrashing frequency if they were not also exposed to AuNPs. In other words, the worms exposed to solely H_2O_2 had significantly lower thrashing rates ($\mu = 30.8$, $SE = 6.8$) than the control group ($\mu = 59.3$, $SE = 2.6$), $F(1,29) = 4.19$, $p = 0.05$, while the locomotion of worms exposed to both AuNPs and H_2O_2 did not differ significantly from the control group ($p > 0.05$). This interaction is shown below in Figure 3.

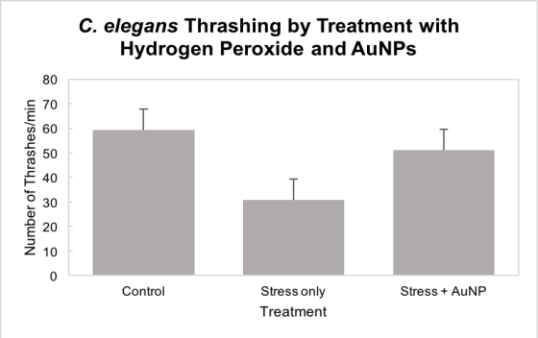

Figure 3. Average Thrashing Frequencies of Three Treatment Groups of *C. elegans*: worms exposed to both AuNPs and H_2O_2-induced stress; only H_2O_2-induced stress; and neither (control). Worms in the control group thrashed 59.3 ± 5.1 times/min on average; worms exposed to only H_2O_2 averaged 30.8 ± 20.3 thrashes/min; and worms exposed to both AuNPs and H_2O_2 thrashed an average of 51.1 ± 30.3 times/min. $p = 0.05$*

A one-way ANOVA was conducted to assess the significance of thrashing data collected in the second experiment. It was found that the relationship between AuNP exposure and locomotion was not significant ($p > 0.05$).

Locomotion by AuNP Exposure Method

Thrashing data collected in the first and second experiments in the present study is shown in Table 3.

AuNP Exposure	Thrashes/Minute
50% K-Medium	46.3 ± 18.4
E. coli OP50	119.6 ± 32.6

Table 3. Average *C. elegans* Thrashing Data by Method of Exposure to AuNPs

No statistical tests were conducted comparing the thrashing frequencies of worms exposed to AuNPs via 50% K-medium or via their food source. But average thrashes were obtained for AuNPs in 50% K-medium ($\mu = 46.3$, $SE = 5.3$) and their bacterial food source ($\mu = 119.6$, $SE = 7.3$).

DISCUSSION

The first experiment in the present study examined the effects of gold nanoparticles on *C. elegans* induced with oxidative stress via exposure to 10 mM H_2O_2. The results of this experiment showed that worms exposed to ROS-induced oxidative stress through H_2O_2 had a higher mortality rate and exhibited delayed reaction to mechanical stimuli when compared to worms that had not been introduced to stress. The significant decrease in mechanosensation suggests that H_2O_2-induced oxidative stress has neurodegenerative effects on mechanoreceptor neurons (MRNs), which is consistent with previous literature findings and provides a baseline for the behavioral comparison of worms solely induced with oxidative stress and worms exposed to both stress and AuNPs.

In addition, *C. elegans* that were introduced to both stress and AuNPs exhibited a significantly higher thrashing frequency than those only exposed to stress. A conclusion can thus be drawn that AuNPs may diminish the negative effects of oxidative stress on *C. elegans*. More specifically, these results suggest that AuNPs may alleviate neurodegeneration at the neuromuscular junction caused by oxidative stress, resulting in the normalized thrashing frequencies observed in worms treated with both ROS and AuNPs when compared to the worms treated with only ROS. It is important to note here that AuNPs have a catalytic effect on the decomposition of H_2O_2.[10] Because of this, future studies should introduce a control that allows the study to determine the actual concentration of H_2O_2 exposed to the worms. Or, by using a completely different agent to induce oxidative stress that does not decompose when introduced to AuNPs, such as paraquat. Following these changes, studies should then be conducted to further investigate whether AuNPs could be a means to relieve the effects of oxidative stress on the neurodegeneration of the NMJ in *C. elegans* by not only assessing neuromuscular activity in thrashing and locomotive assays, but also by quantifying amounts of acetylcholine (ACh), an essential neurotransmitter at the NMJ, before and after exposure to AuNPs. Other data could be collected such as levels of dopamine (DA), serotonin (5-HT), and GABA, antioxidant activity, and other behavioral data such as pharyngeal pumping, mechanosensation in response to food, etc.

Results from this study may also have implications in animals beyond *C. elegans*, and future replicates of this study and similar studies would be encouraged with other invertebrate and vertebrate species. In humans, AuNPs could be explored in the future as a possible therapy for neurodegeneration at the NMJ of people who suffer from amyotrophic lateral sclerosis (ALS), a neurodegenerative disease that affects the muscles and locomotion of patients.

The second experiment showed *C. elegans* that were introduced to gold nanoparticles via their food source (ingestion) were found to have a higher amount of movement than those that were introduced via a liquid medium. While it is possible that the nematodes introduced to the gold nanoparticles through the liquid medium had decreased function at the NMJ because they were deprived of food for 24 hours, further exploring the different techniques by which AuNPs can be introduced to *C. elegans* may give insight into the uptake of gold nanoparticles and their effect at the neuromuscular junction of *C. elegans* worms. This data, while unable to be compared to each other without first being compared to controls respective of their medias, still allows us to draw conclusions about the interaction of AuNPs on *C. elegans.* Future studies in which the introduction of AuNPs to worms via food and vehicle should include a way to measure the concentration of H_2O_2 exposed to the worm, as well as those that explore whether these positive outcomes were due to concentration differences, uptake efficiency, or other factors by including a control per media used.

Prior to the start of the experiment, it was predicted that AuNP exposure would have a significantly positive effect on *C. elegans* mechanosensation, locomotion, and survival; that H_2O_2 would have a significantly negative effect on *C. elegans* mechanosensation, locomotion, and survival; and that *C. elegans* exposed to both

AuNPs and oxidative stress would not experience the same decrease in mechanosensation, locomotion, and survival predicted for worms exposed only to H_2O_2. The first prediction was not supported by the data, as AuNPs were not shown to have any significant effect on mortality, mechanosensation, or thrashing frequency when compared to the control. The second prediction was supported by the data and has been supported by numerous studies published prior to the start of this study, as H_2O_2 is an ROS proven to induce oxidative stress in *C. elegans* and a number of other species. The third prediction was also supported by the data, as a significant interaction between the AuNP and H_2O_2 exposure variables was observed, suggesting possible reversal of the neurodegenerative effects of ROS-induced oxidative stress by AuNP exposure. This finding provides a basis into future research into the therapeutic properties of gold nanoparticles, specifically in reference to oxidative stress and neurodegeneration of the neuromuscular junction.

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