Effect of increasing hydrogen peroxide concentrations on head oscillations of *Caenorhabditis elegans*

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Abstract

Hydrogen peroxide is commonly used as a disinfectant and bleaching agent. In this study, we observed the effect of hydrogen peroxide on the locomotive ability of *Caenorhabditis elegans*. We used four treatment levels, consisting of three different concentrations of hydrogen peroxide solutions at 1%, 2% and 2.5%, and a storage buffer (0%) as the control. We used a DinoXcope to record a video of the initial 30 seconds of exposure to 2 μL of each solution, we then used these videos to count the number of head oscillations that occurred within this time. The mean head oscillations measured at 0%, 1%, 2% and 2.5% hydrogen peroxide were 5 ± 3, 87 ± 11, 74 ± 8, and 72 ± 7, respectively. According to our one-way ANOVA calculation, the measured oscillations showed a statistically significant difference between the number of head oscillations in the hydrogen peroxide treatments and that of the control treatment. Increasing hydrogen peroxide concentration has an effect on the number of head oscillations of *Caenorhabditis elegans* ($p = 8.4116 \times 10^{-15}$). Hydrogen peroxide may have had an effect on the ASH chemosensory neurons and LITE-1 gustatory receptors which caused the observed increase in head oscillations as an attempt to escape from exposure.

Introduction

Caenorhabditis elegans is a small free-living nematode with an average length of about 1 mm (Portegys 2015). Each nematode has an intricately mapped nervous system comprised of 302 neurons and 3,680 unique synaptic connections (Portegys 2015). A large portion of this nervous system is used as a tool for recognizing compounds in their surrounding environment (Bargmann 2006). *C. elegans* has a fully functioning chemosensory system that uses chemosensation to identify potential danger, find food and locate mates (Bargmann 2006). To sense chemicals, 32 chemosensory neurons reveal their sensory cilia to the environment (Bargmann 2006). They do this by piercing through the thin tissue of their exoskeleton, in three different openings: the amphid, plasmid and inner labial organs (Bargmann 2006, Ward 1973).

These chemosensory neurons are believed to enable an overall physical response from exposure to chemical compounds, which, in this case can be produced by hydrogen peroxide, an oxidizing agent that freely mixes with water and easily passes through cellular membranes (Halliwell *et al.* 2000).

In natural environments *C. elegans* can be exposed to hydrogen peroxide through rainwater, production by bacteria, such as *Streptococcus* and *Lactobacillus,* and production by bombardier beetles (Kiontke and Sudhaus 2006, Bhatla and Horvitz 2015). Although evidence suggests that there is an effect of hydrogen peroxide on chemosensory neurons and gustatory sensation, the particular mechanisms enabling a response within the nervous system and gustatory system, taste detecting system of chemical compounds, are not fully understood (Bargmann 2006, Bhatla and Horvitz 2015).

The ability of *C. elegans* to detect and respond to chemical stimulants by rapid withdrawal from the original source is due to the presence of LITE-1 (high-energy light unresponsive) gustatory receptors, which detect specific flavours, and also ASH (Amphid Sensory neuron class H) chemosensory neurons. These neurons are similar to pain sensing neurons in vertebrates and cue physical movement responses using voltage-activated calcium channels (Figure 1).

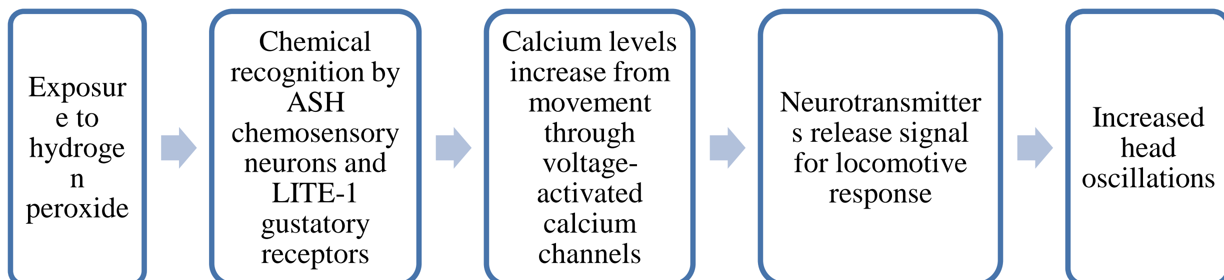

Figure 1. Proposed model for effect of hydrogen peroxide: Hydrogen peroxide exposure enables chemical recognition by ASH chemosensory neurons and LITE-1 gustatory receptors which increase the calcium levels from movement through voltage-activated calcium channel. As a result, neurotransmitters respond by signaling a locomotive response away from the chemical source causing increased head oscillations.

We modeled our experiment on the study by Caldwell *et al.* (2003), which studied the effects of chemical sanitizers on the recovery and reproductive behaviour of *C. elegans*. Caldwell *et al.* (2003) used treatment concentrations of 0.5%, 1% and 2% hydrogen peroxide as a form of a chemical sanitizer. The 0.5% and 1% treatments had no effect on the recovery and reproductive behaviour of *C. elegans*. However, the 2% treatment resulted in death after one day (Caldwell *et al.* 2003). Based on their results, we tested the effect of hydrogen peroxide concentrations on the head oscillations of *C. elegans* during direct exposure to similar concentrations of 1%, 2% and 2.5% of hydrogen peroxide. Using these findings, we predicted that increasing hydrogen peroxide concentrations will increase head oscillations in *C. elegans* as they attempt to escape the chemical medium. We formulated the following hypotheses:

H_o: Increasing hydrogen peroxide concentrations have no effect on the number of head oscillations of *C. elegans.*

H_a: Increasing hydrogen peroxide concentrations have an effect on the number of head oscillations of *C. elegans.*

Methods

We selected N2 wild type *C. elegans* for this experiment, each adult was approximately 1 mm in length. The accompanying equipment used in our experiment were Kyowa dissecting microscopes, DinoXcopes (Dino-Lite Digital Microscopes), a storage buffer solution (21.25 mL of 0.15 M K_2HPO_4, 21.775 ml of 1 M KH_2PO_4, and 2.93 g NaCl), 2.5% hydrogen peroxide solution, 60-mm Petri dishes with agar, and counters.

We tested the nematodes in four different treatments for this experiment. Three experimental treatments solutions consisted of 1% hydrogen peroxide, 2% hydrogen peroxide,

and 2.5% hydrogen peroxide, whilst the control treatment solution consisted solely of storage buffer. The 1% and 2% hydrogen peroxide were diluted from a 2.5% hydrogen peroxide stock solution with the storage buffer. For each of the four treatments, there were eight replicates.

Our setup consisted of a DinoXcope attached to a Kyowa dissecting microscope, at 7X magnification, for video-recording of each replicate (Figure 2). For each replicate, we extracted a nematode of appropriate size from the population provided in a Petri dish with *Escherichia coli,* a primary food source for *C. elegans*. We then transferred the nematode to the centre of a clean Petri dish, pre-inoculated with *E. coli*, on the stage of the Kyowa dissecting microscope using a platinum pick. During the transfer, we used proper sterile techniques to avoid any possible contamination.

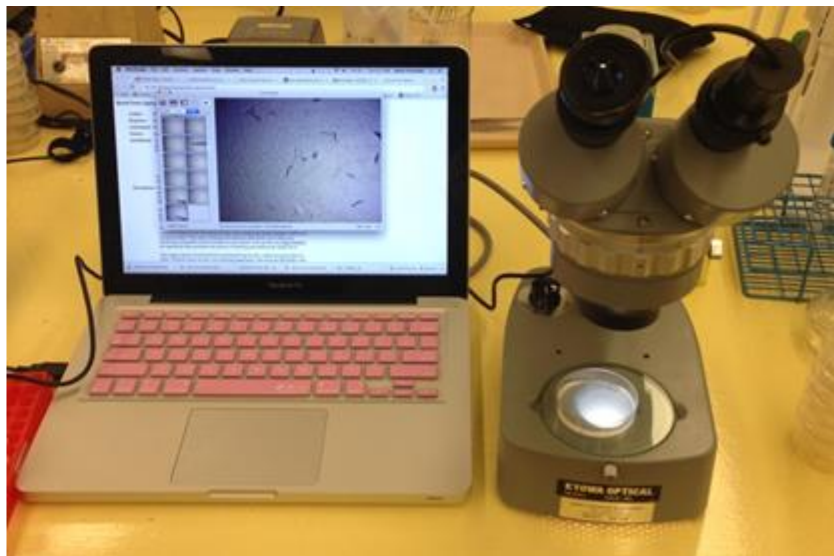

Figure 2. The standardized setup of our experiment including the Kyowa dissecting microscope, the DinoXcope connecting the microscope to laptop for video recording, and a Petri dish placed on the stage of the microscope.

Based on a pilot study, we placed a 2-µL drop of solution, corresponding to the test treatment level, on top of the nematode using a micropipette (Figure 3). We found that 2-µL droplets fully submerged the nematodes in solution and dried up before causing detrimental effects, such as complete impairment on the nematode's mobility.

Immediately after initial chemical exposure, we recorded the nematode's movement using a DinoXcope for 30 seconds (based on time constraints and the rapid evaporation rate of treatment droplets). We recorded head oscillations within the first 30 seconds of chemical exposure for all treatments.

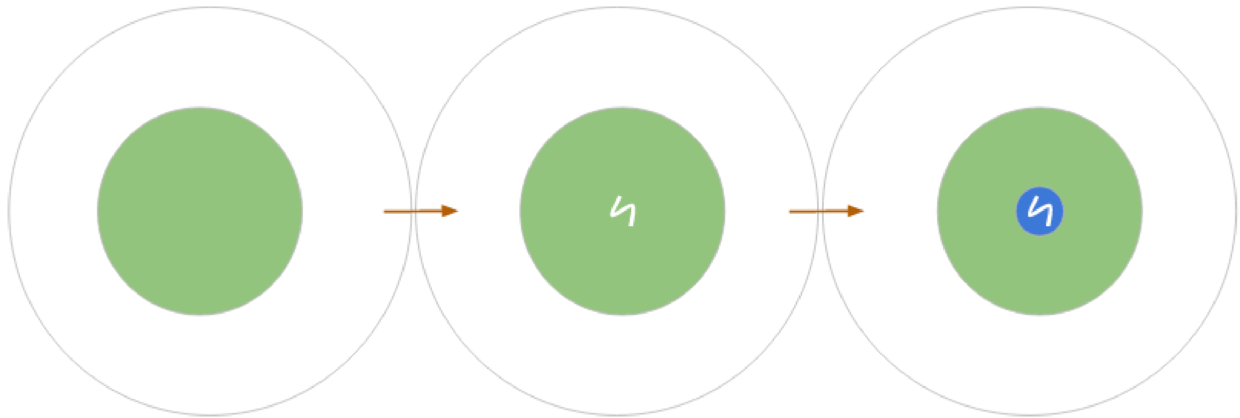

Figure 3. View overlooking the agar plates. The green colour is the agar with *E. coli*, at the centre is the droplet of hydrogen peroxide treatment solution (blue) with a single *C. elegans* inside.

When analyzing our data, we counted one head oscillation each time the head of the *C. elegans* crossed the 90° line, from the anterior region to the posterior region of the body (Figure 4). To ensure each head oscillation was measured consistently, an experimenter counted while reviewing the recorded video at half-speed. The experimenter repeated measurements three or more times in random order, with the counter covered, in order to minimize bias and deviation. The counted values obtained from each replicate were then averaged, rounded to whole numbers, and used for further statistical analysis. We calculated the overall means, standard deviations and 95% confidence intervals of head oscillations at each treatment level. We then conducted a one-way analysis of variance (ANOVA).

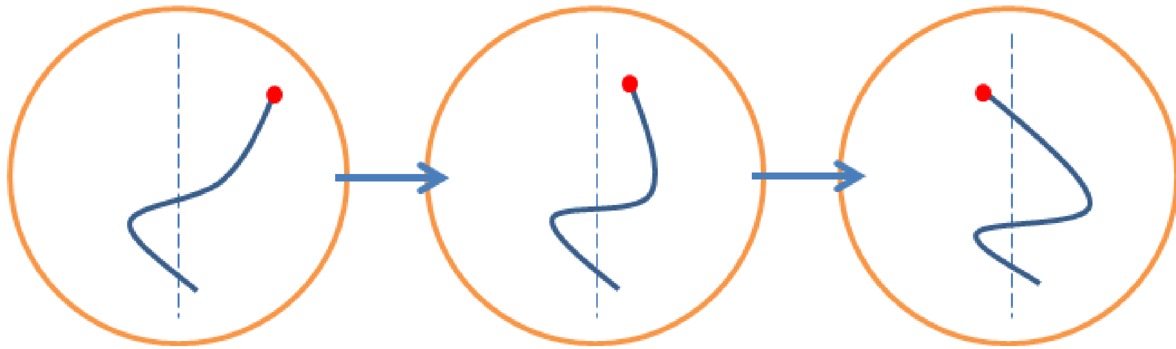

Figure 4. As the head (red) of the nematode crosses the 90° vertical line (dashed) once, one head oscillation would be counted.

Results

Initial observations indicated that *C. elegans* typically move their heads in smooth oscillating motions. During exposure to the control treatment (buffer), *C. elegans* continued to exhibit normal head oscillations (Figure 5). However, when the nematodes were exposed to hydrogen peroxide, head oscillations became rapid and non-uniform. Additionally, head oscillations appeared more vigorous in lower concentrations of hydrogen peroxide, the most vigorous movements were observed in the 1% hydrogen peroxide treatment (Figure 5) and the least vigorous in the 2.5% treatment. Although the *C. elegans* oscillated vigorously, many of them oscillated in the same spot during the observed period. Furthermore, the number of head oscillations that occurred directly after exposure decreased slightly as concentrations of hydrogen peroxide increased from 1% to 2.5%.

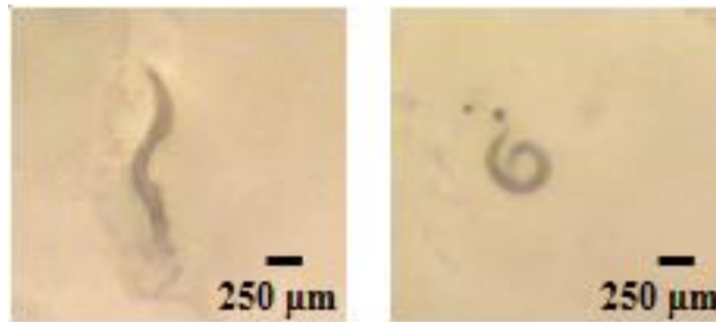

Figure 5. A comparison between the shapes of the *C. elegans* in the control treatment (left) and 1% hydrogen peroxide treatment (right) show that the nematodes curl up as they oscillate in a toxic environment, whereas the nematode of the control remains in an extended S-shape.

The mean head oscillations of *C. elegans* measured in the control treatment (0%), 1%, 2%, and 2.5% treatments of hydrogen peroxide were 5 ± 3, 87 ± 11, 74 ± 8, and 72 ± 7, respectively (Figure 6). Mean head oscillation measurements and 95% confidence intervals were plotted. A *p*-value of 8.4116×10^{-15} (one-way ANOVA) was calculated. Upon further analysis, we observed a slight negative trend in the number of head oscillations as concentrations of hydrogen peroxide increased from 1% to 2.5% (Figure 6).

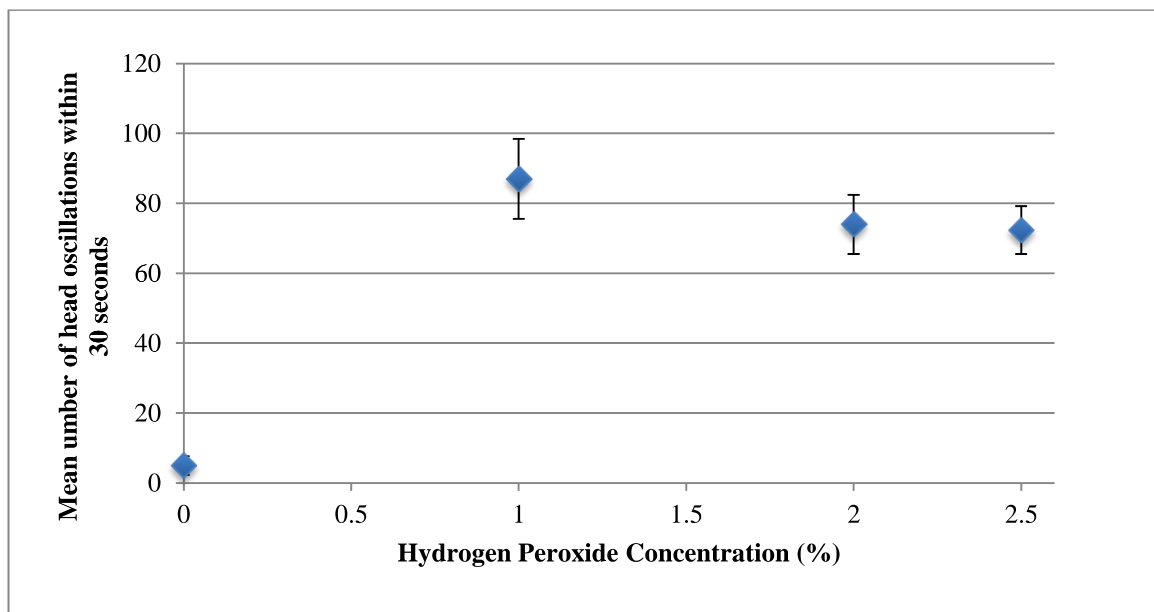

Figure 6. The effects of varying concentrations of hydrogen peroxide on the average number of head oscillations in *Caenorhabditis elegans* within 30 seconds after initial exposure. Treatments consisted of a 0% control with buffer, 1%, 2% and 2.5% hydrogen peroxide. Error bars indicate 95% confidence intervals, $p = 8.4116 \times 10^{-15}$ ($n = 8$).

Discussion

After carrying out a one-way ANOVA on the mean head oscillations per treatment level, we rejected the null hypothesis and provided support for the alternative hypothesis ($p < 0.05$). Based on these statistical results, we were also able to support our prediction that increasing hydrogen peroxide concentrations increase the number of head oscillations of *C. elegans*.

As seen in Figure 6, there were significantly more mean head oscillations in the 1%, 2% and 2.5% hydrogen peroxide treatments than the control treatment, 0% hydrogen peroxide. However, after exposure to 1% hydrogen peroxide, the number of head oscillations decreased slightly in the 2% and 2.5% treatments. The ability of hydrogen peroxide to easily and readily permeate cellular membranes may be a cause for the observed negative trend (Halliwell *et al.* 2000). This increases intracellular hydrogen peroxide, which leads to a decrease in ATP within the cell (Baker *et al.*1989). This can be attributed to the oxidation of the glyceraldehyde-3-phosphate dehydrogenase protein, which reduces the synthesis of ATP in glycolysis (Baker *et al.* 1989). Therefore, the nematode will tend to oscillate less as there is a decreased amount of ATP available.

Near the end of the observed 30 seconds, *C. elegans* decreased their movement and transitioned into a still, sleep-like state. Hill *et al.* (2014) describes this inactive behaviour as a transitional quiescent state, a sleep-like period of inactivity which takes place after exposure as a survival tactic against cellular stress. This allows resources designated for regular cell processes, such as mobility and reproduction, to shift in the direction of initiating cellular responses for recovery from potential harm (Hill *et al.* 2014).

C. elegans that were exposed to hydrogen peroxide exhibited quick and exaggerated body movements, whereas *C. elegans* treated with storage buffer moved in a slower S-shaped fashion.

A biological mechanism that may explain the violent oscillations of the hydrogen peroxide treated nematodes is the binding of LITE-1. The LITE-1 is uniquely classified as a gustatory receptor, which has the ability to detect a variety of specific flavours (Bhatla and Horvitz 2015). Salty, sweet and savory flavours typically indicate a non-threatening compound that can be ingested, whereas, bitter and sour flavours indicate a toxic compound that is immediately avoided (Bhatla and Horvitz 2015). The response observed when the *C. elegans* are exposed to a large amount of hydrogen peroxide may be analogous to a bitter sensation in mammals with functioning taste receptors. The exact mechanism for LITE-1 action is unknown. However, it is a receptor believed to detect threat by binding with reactive oxidative species, such as hydrogen peroxide, to promote a locomotory avoidance response (Bhatla and Horvitz 2015).

Bargmann (2006) also describes the rapid and erratic body movement of the *C. elegans* as a withdrawal response from exposure to chemical repellents. Two important components that are required for the chemorepulsion responses are the ASH chemosensory neurons and the phasmid neurons. Phasmid neurons are sensilla, simple sensory receptors that are located in the tail of *C. elegans* (Bargmann 2006). Phasmid neurons can block ASH neuronal functions through synaptic processes (Bargmann 2006). By controlling the neuronal functions of ASH neurons, the phasmid neurons can regulate the directional movement in the *C. elegans*' chemorepulsion response and propel *C. elegans* in a desired direction away from the chemical source (Bargmann 2006). The phasmid neuron's ability to block the function of ASH chemosensory neurons also suggests that *C. elegans* are able to sense chemical repellents with their head and tail (Bargmann 2006). Therefore, the observed wide head movements and sudden changes in direction may be the nematode’s attempt to find a region with a lower concentration of the chemical.

When a chemical repellent is introduced to *C. elegans*, the ASH neurons may induce a sharp increase in their neuronal calcium concentration (Bargmann 2006). As seen in Figure 1, a large influx of calcium ions through voltage-activated calcium channels into neuronal pathways enables neurotransmitter release, which then causes rapid depolarization of the ASH neurons. This may have resulted in the observed increase of head oscillations in hydrogen peroxide-exposed *C. elegans* (Bargmann 2006).

In the experiment conducted by Caldwell *et al.* (2003), effects of hydrogen peroxide exposure were observed for four days; in our experiment the effects of exposure were observed for 30 seconds. Our experiment specifically observed head oscillations as a result of hydrogen peroxide exposure, whereas, Caldwell *et al.* (2003) observed reproduction and viability. In spite of different methods used, our results are comparable with their results.

An aspect of uncertainty in our experimental design was the method of which head oscillations were counted. For instance, the head oscillations measurements recorded were manually counted using a 90° line through the frontal and posterior region of the nematode as a reference, previously shown in Figure 4. Although we used this counting method as a guideline, there were occasions where it was difficult to count precisely due to *C. elegans* exhibiting rapid successive oscillations. Another source of uncertainty in our experiment was the biological variations of the nematodes tested. We chose nematodes of with the length of around 1 mm to be tested, however, the variation in size could have affected their ability to react with the hydrogen peroxide. For example larger nematodes may have had a higher tolerance than smaller nematodes; therefore, our results may only represent a small portion of the general wild-type population.

Conclusion

Our experiment tested the response of *C. elegans* when exposed to hydrogen peroxide by observing the number of head oscillations for the first 30 seconds of chemical exposure. Based on our one-way ANOVA statistical results, we were able to reject our null hypothesis and provide support for our alternative hypothesis: increasing hydrogen peroxide concentration has an effect on the number of head oscillations of *C. elegans.* The information on the effects of chemical exposure that this study provides can be further applied to other nematodes. This may, therefore, increase understanding chemosensation in free-living worms.

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