Oxidative damage induced by hydrogen peroxide is counteracted by the antioxidant enzyme catalase.

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that impairs the motor neurons, thus resulting in a progressive inhibition of voluntary muscle movements (Rowland, 2001). Oxidative stress has been established as an essential mechanism in the pathogenesis of ALS (Labo et al., 2010). The aim of this experiment was to induce oxidative stress in the crayfish neuromuscular junction by adding hydrogen peroxide (H_2O_2) to observe the potential antioxidant action of catalase. We first predicted that exposure of the crayfish neuromuscular junction to H_2O_2 would decrease excitatory junction potential (EJP) amplitudes over time. We then hypothesized that upon addition of catalase, the decrease in EJP amplitudes produced due to H_2O_2 exposure would be prevented. The experiment involved isolating the superficial extensor muscle in the crayfish tail, submerging it in solutions of normal saline, H_2O_2, and then H_2O_2 and catalase together. Data collection involved delivery of an electrical impulse to stimulate the nerve innervating a muscle cell and taking intracellular recordings, which could then be used to calculate EJP amplitude. We found that H_2O_2 exposure resulted in a decreased EJP amplitude and catalase successfully prevented this change.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease. The disease is progressive, meaning its symptoms worsen over time (Rowland, 2001). There is currently no cure for ALS and very few treatments exist that are successfully able to prevent its symptoms. ALS impacts primarily the motor neurons, which are the nerve cells responsible for controlling voluntary muscle movement. In ALS, both the upper motor neurons, located in the motor region of the cerebral cortex or the brainstem, and the lower motor neurons, located in the ventral horn of the spinal cord gray matter and the motor nuclei of the cranial nerves in the brainstem, degenerate and eventually die (Purves, 2001). This impairment prevents messages from being sent to muscles (Hardiman et al., 2011), which is why ALS patients experience both muscle weakness/deterioration and paralysis (The ALS Association, 2017).

Previous research has found that a contributor to the causes of ALS is oxidative stress. Oxidative stress is the imbalance between the production of free radicals in the body and its ability to efficiently break them down to detoxify their harmful effects (Mandal, 2017). The most common types of free radicals are reactive oxygen species (ROS), which are produced naturally in the body (Howard, 2017). Increased production of ROSs and oxidative damage to proteins and lipids have both been found in ALS patients (Liu et al., 2015). Hydrogen peroxide (H_2O_2) is a primary reactive oxygen species involved in causing oxidative stress. Antioxidants are able to stop this chain reaction of oxidative stress by donating one of their own electrons to stabilize the free radicals, without becoming free radicals themselves (Labo et al., 2010). Catalase is a known antioxidant enzyme that decomposes H_2O_2 into two stable molecules: water (H_2O) and oxygen (O_2), thereby protecting cells from oxidative stress caused by H_2O_2.

Scientists are currently investigating the effects of different molecules on oxidative stress. Bukharaeva et al. (2015) investigated the effect of homocysteine on oxidative stress-induced impairment of neurotransmitter release by using a mouse diaphragm muscle. In addition to using homocysteine, these scientists also found that combining homocysteine and H_2O_2 enhanced oxidative stress. This experiment, however, did not focus on how only H_2O_2 contributed to oxidative stress. Therefore, we decided to investigate further into the impact of specifically H_2O_2 and propose potential ways to prevent oxidative stress caused by H_2O_2. Another experiment conducted by Babu et al. (2008), found that in places of low concentration of H_2O_2 there were higher concentrations of catalase. Babu et al. concluded that there was a low H_2O_2 concentration in these areas because catalase was successfully breaking down the molecule (Babu, 2008). However, Badu et al. had not connected the breakdown of H_2O_2 by catalase to the context of counteracting neuron degeneration caused by oxidative stress in ALS patients. Using the results from Bukharaeva et al. and Badu et al., we constructed an experiment centered around the interaction of oxidants (H_2O_2) with antioxidants (catalase) in the context of ALS and the neuromuscular junction in crayfish. In order to investigate

whether this decomposition reaction (treatment) works we created an experiment that simulated the conditions of ALS (oxidative stress) on crayfish neuromuscular junction.

We hypothesized that H_2O_2 would initially decrease the amplitude of EJPs in the crayfish, but that this decrease would be prevented upon addition of catalase. We investigated this hypothesis by using intracellular recording and nerve stimulation to compare the EJP amplitudes created in Ringer's saline solution (control), H_2O_2 solution, and H_2O_2 + catalase solution. We found that when the crayfish was incubated in a solution with catalase and H_2O_2 there was no decrease in EJP amplitude, therefore supporting our hypothesis.

MATERIALS AND METHODS

Organism

The crayfish was first anesthetized via an ice bath. We then removed the tail, which was the only part of the crayfish we used. We made two incisions along the sides of the tail so that the ventral side of the abdomen could easily be removed. We carefully used our thumb to remove the large mass of muscle to expose the underlying extensor muscle cells and nerves, after which we pinned the tail to a petri dish and submerged it in Ringer's saline solution so as to keep the cells alive (Wyttenbach, et al., 1999).

Electrophysiology

We used intracellular recording with glass microelectrodes. We used two electrodes: one to measure the EJPs by inserting it into the muscle cell and another one with a larger point diameter to suction a nerve. We created the electrodes by taking a capillary tube of diameter 1.2mm, and placing it into the holders of the pipette puller. The electrodes we inserted into the muscle cells were filled with 3M KCL solution. These electrodes had a resistance of 4-20MΩ. While we filled the electrode with the KCl, it was very important that no bubbles were formed, otherwise the level of resistance and therefore reliability of results would have been impacted. We then placed the filled electrode into an electrode holder. To make the electrode to suction the nerve, we filed down the tip of the electrode to make the tip diameter large enough to hold a nerve, but still small enough to keep the nerve inside the electrode.

Solutions

For our control condition we submerged the crayfish tail in Ringer's saline solution (5.4mM KCL, 196mM NaCl, 2.6mM $MgCl_2 \cdot 6H_2O$, 10 mM Hepes buffer, 13.5mM $CaCl_2 \cdot 2H_2O$). For one of the experimental conditions, we incubated the crayfish in a solution of H_2O_2 (330μM) to test the effect of H_2O_2 alone on EJP amplitudes. For another condition, we incubated the crayfish in a solution containing H_2O_2 (330μM) and catalase (25,000 units/liter). The catalase concentration was taken from a prior experiment conducted by Milton (2008). We left the muscle cells in each of these solutions for 40 minutes. We stimulated the nerve cell and recorded the amplitude of the EJPs in intervals of 5 minutes.

Data collection

We placed the crayfish under a microscope to make it easier to accurately position the electrode inside the muscle cells. Another electrode was used to suck up a nerve cell in order to stimulate it with a voltage ranging between .16-.4 volts. Once the nerve cell was stimulated, the electrode that was inserted into the muscle cell recorded the EJP.

Raw data calculations

Our raw data consisted of 5 samples of the resting potential and 5 samples of the maximum potential upon administering the stimulus in intervals of 5 minutes. We calculated the amplitude of the EJPs for each of these 5 samples by subtracting the initial resting membrane potential (minimum point on the graph) from the peak of the curve (maximum point on the graph). For the condition with only H_2O_2 as well as the setting with both H_2O_2 and catalase, 5 pulses were delivered every 5 minutes over a total period of 40 minutes, such that 5 measurements of amplitude were taken at each interval. These 5 amplitudes were averaged to calculate the mean EJP at each time interval. The same procedure was applied to the calculations for the remaining settings.

Data Analysis

Because we compared different treatments to see whether or not they cause a significant change in the amplitude of the EJP produced, we used multiple T-tests. One T-test compared the control data with the H_2O_2 + catalase treated data to verify statistically that the catalase caused no decrease in EJP amplitude. Another T-test compared the H_2O_2 treated data to the data set treated with both H_2O_2 and catalase, which enabled us to verify if the addition of catalase significantly reduces the detrimental effect of H_2O_2 on EJP amplitude. We also calculated the percentage change between 0 min and 40 min in the H_2O_2 + catalase condition.

RESULTS

(a)

(b)
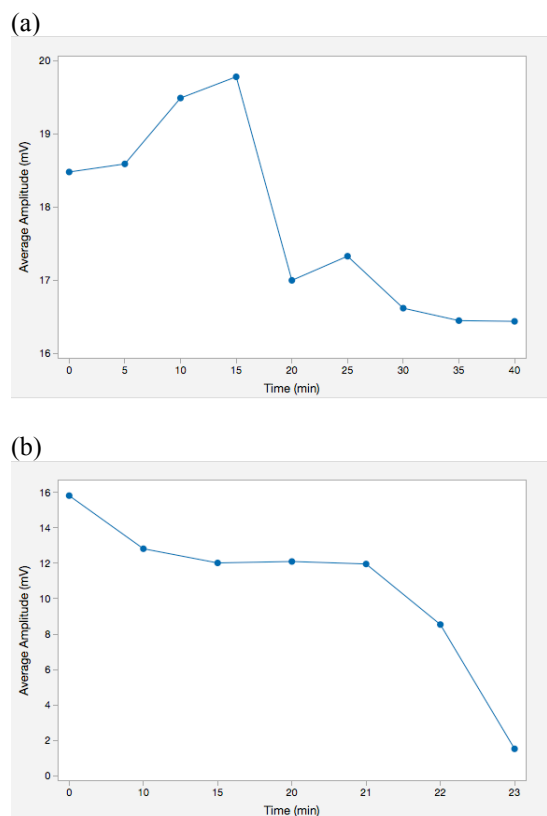
(data collected by Andrew Tucker, Heather Suh, Rachel Buckner)

Figure 1. The effect of H$_2$O$_2$ on the amplitude of excitatory junction potentials (EJP) produced in crayfish extensor muscle cells for two different trials. Panels (a) and (b) represent two different trials conducted with H$_2$O$_2$. The data points represent the average amplitude of 5 samples of the EJPs measured at the indicated time interval following exposure to H$_2$O$_2$ at T= 0 minutes.
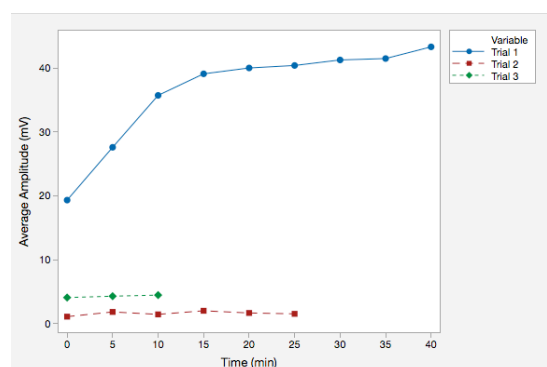

Figure 2. The average amplitude of EJPs in a H$_2$O$_2$ + catalase solution in relation to time, compared across three different trials. Trial 1 was recorded for 40 minutes, Trial 2 was recorded for 25 minutes, and Trial 3 was recorded for 10 minutes. The data points represent the average amplitudes of the EJPs, from 5 samples, at the indicated time. Trial 1 was recorded for 40 minutes, Trial 2 was recorded for 25 minutes, and Trial 3 was recorded for 10 minutes.
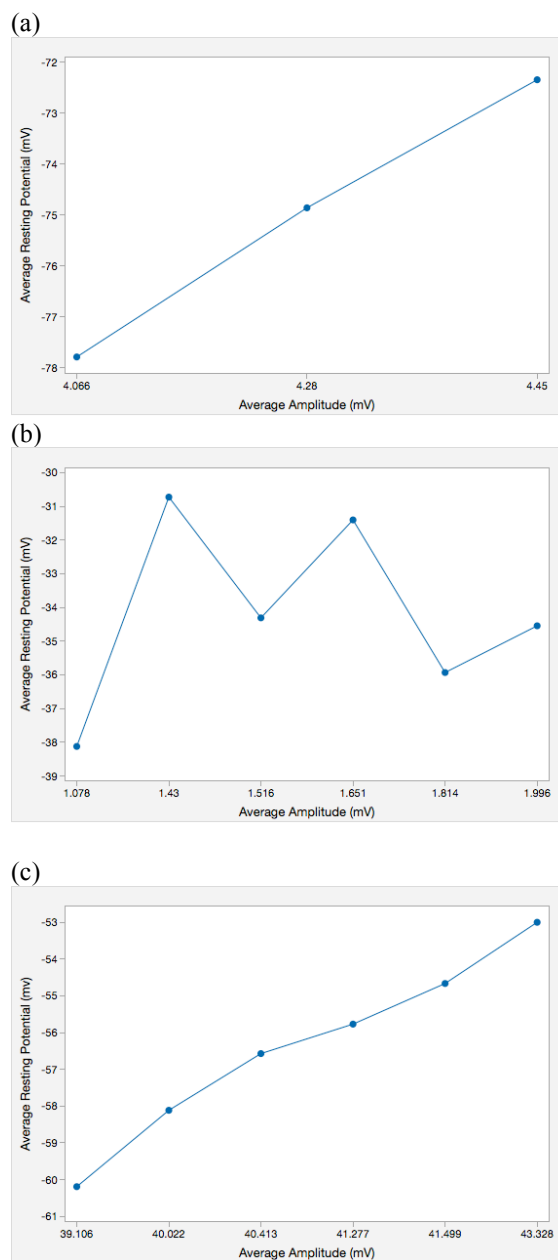
(a)

(b)

(c)

Figure 3. The relationship between the average EJP amplitude and resting potential when muscle cells were incubated in a H$_2$O$_2$ + catalase solution. (a)Trial 1 was recorded for 40 mins in solution, (b) Trial 2 was recorded for 25 mins in solution, (c) Trial 3 recorded for 10 mins in solution. The points on the graph represent the average resting membrane potential, from 5 data samples, in relation to the average EJP amplitude of 5 samples as time progresses.

The goal of this experiment was to investigate whether an antioxidant like catalase could prevent the decrease in EJPs caused by the oxidant, H$_2$O$_2$. We investigated this mechanism of action of ALS by comparing the change in EJP amplitude after exposing the crayfish to increased levels of H$_2$O$_2$ and then H$_2$O$_2$ + catalase. Past experiments have found that H$_2$O$_2$ decreases the amplitude of the EJPs

(Rowland, 2001). We therefore tested catalase (antioxidant) as a potential solution to the negative effect of H_2O_2 on motor neurons. With the help of intracellular recording and nerve stimulation we caused an action potential in the nerve and then recorded the resulting EJP amplitudes.

When the crayfish motor neurons were exposed to H_2O_2 there was a decrease in the EJP amplitudes (Figure 1). Without the addition of H_2O_2 the average amplitude of the EJPs was 18.49mV. Upon exposure to H_2O_2 (at time 0 min) the amplitude of the EJPs decreased, starting at 20 minutes of incubation. After 40 minutes of exposure the EJP amplitudes had decreased to 16.44mV. The data in panel (b) was collected from a fellow research group (Andrew Tucker, Heather Suh, and Rachel Buckner). Their data shows a similar trend in the decrease of EJPs after the hydrogen peroxide was added. The initial average amplitude of the EJPs was 15.82mV. After 23 minutes the EJP amplitudes had decreased to 1.52mV. Over an incubation period of 23 minutes with H_2O_2, the average EJP amplitudes decreased by 13.76 mV (from 15.28 to 1.52). From this decrease, we can infer that the oxidative stress induced by addition of H_2O_2 impaired the neuromuscular junction and inhibited the production of larger EJPs. Our results from our H_2O_2 test were consistent with previous research.

In the H_2O_2 + catalase solution, we did not observe any decrease in EJP amplitudes, which is different from the decrease we saw when the tail was submerged in a H_2O_2 solution (Figure 2). Trial 2 began with an average amplitude of 1.1mV and ended with an average of 1.516mV, showing a minimal increase of 0.3mV. Trial 3 began with an average amplitude of 4.1mV and ended with 4.5mV also showing an insignificant increase of 0.4mV. Trial 2 and 3 showed a minimal increase in the average amplitude but mostly stayed the same over the course of the incubation period, and neither trial showed a decrease in average amplitude (Figure 2). To the contrary, in trial 1 the initial average amplitude was 19mV and by the end of the 40-minute incubation period the average amplitude had increased to 43mV showing a 24mV increase in the average amplitudes. Each of these trials used a different crayfish, meaning that despite variation in individual organisms, the eventual trend we found is a constant because 3 separate crayfish showed no decrease in EJP amplitude when in a solution of both H_2O_2 and catalase. We found no significant difference between the control experiment to the experimental condition with both H_2O_2 and catalase (T=-1.05, P= 0.4031). The results support our hypothesis because we predicted that the addition of catalase would prevent the H_2O_2 from significantly altering the amplitudes of the EJPs. We also found no significant difference between the experimental condition with only H_2O_2 to the experimental condition with both H_2O_2 and catalase (T =-1.68, P= 0.3418). This lack of significance was not as predicted, and may be due to the limited number of trials we have for this specific setting.

Upon conducting our experiment, we noticed an unpredicted relationship between the value of the resting potential and the calculated EJP amplitudes. As the EJP amplitude increases, the resting potential also increases (becomes less negative) resulting in a positive relationship. Trials 1 and 3 both have more linear trends, whereas trial 2 showed a much subtler positive trend with several fluctuations (Figure 3).

DISCUSSION

The results of this experiment supported our hypothesis that H_2O_2 would initially decrease the amplitude of the crayfish EJPs but with the addition of catalase it would prevent the effects of H_2O_2. The H_2O_2 caused the EJPs to decrease when added to the crayfish. When the catalase was added with the H_2O_2 there was no longer a decrease in the EJP amplitudes. Trials 2 and 3 displayed similar EJP amplitudes to the control experiment. Although the data presented a visual difference (Figure 2) between the H_2O_2 only condition and the H_2O_2 plus catalase condition, there was no statistically significant change.

These results could have been caused by a few reasons. In reference to the H_2O_2 plus catalase results, the catalase effectively broke down the H_2O_2 molecules into water and oxygen molecules. Therefore, the H_2O_2 was unable to decrease the EJPs produced by the crayfish muscles neurons because it stopped it from causing oxidative stress (Rowland, 2001). The effectiveness of the catalase was in agreement with the experiment conducted by Babu (2008), who discovered that ALS patients who showed less signs to oxidative stress had an increase in the enzyme catalase because it plays a big role in reducing oxygen intermediates.

In the first trial of the H_2O_2 plus catalase condition there was a significant increase in the EJP amplitude and resting potential as time progressed (Figure 2). This unanticipated result could be explained by an undetected effect of only catalase on the muscle cells. Due to these results, it would be beneficial, to research the effect of only catalase on the muscle cells.

Our 3 trials of the H_2O_2 plus catalase condition showed a significant difference from the results found from the H_2O_2 condition; however, there was no significant difference in the data. This insignificant difference can be explained by the lack of trials that were successfully carried out during the experimentation period. If this experiment could be re-done we

recommend a greater number of trials for all the conditions in order to find more significance.

There were some difficulties and limitations that potentially affected our data. First, our lab research consisted of multiple lab days, which means our day to day data collection varied, which can cause an inconsistency with specific data points. By conducting a control treatment (only normal Ringer's saline solution) at the beginning of each lab day with every new crayfish we were able to resolve some of this variability. In addition, for our experiment we used an invertebrate instead of a vertebrate therefore the effects of catalase may differ when applied to humans. If the catalase effectively reduces oxidative stress then the next step would be to try it on a simple vertebrate, such as a mouse. Our experiment also has some limitations, for example, with the currently available equipment we are unable to detect other factors that might influence the interaction between H$_2$O$_2$ and catalase. Identifying and isolating other external influences would make for a good future investigation. Another way to rule out the effect of just catalase on synaptic transmission would be to measure EJP amplitudes in crayfish muscle cells incubated in a Ringer's saline solution with only catalase added to it.

In addition to increasing trials to improve significance and testing catalase on the muscle cells, it would be interesting to test whether catalase can reverse the effects of H$_2$O$_2$. In this case, would catalase be effective even after oxidative stress has damaged the motor neurons? An important investigation could come out of this because many ALS patients have already undergone major oxidative stress damage by the time they are looking for treatment options. Another possible future investigation would be to determine the effectiveness of catalase at different concentrations with its ability to prevent H$_2$O$_2$ from causing oxidative stress.

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