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Glutathione and Coenzyme Q10 Show No Additive Neuroprotective Effect at the Neuromuscular Junction of Crayfish

ALEX ROSS, and HARPER SMITH
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

This study examined the effects of antioxidants Coenzyme Q10 and Glutathione at the neuromuscular junction of crayfish. We hypothesized that a solution containing both Coenzyme Q10 and Glutathione would show higher EJPs then each of the antioxidants independently. Microelectrodes were inserted into the superficial extensor muscles of a crayfish tail to record the excitatory junction potential (EJP) of crayfish exposed to three different combinations of antioxidants in response to a neurodegenerative substance (hydrogen peroxide). We found that there was no significant additive effect when Coenzyme Q10 and Glutathione are applied together on preventing neuromuscular degeneration.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a complex neurodegenerative disease that affects some 20,000 Americans each year (The ALS Association, N.D.). ALS patients suffer from the degeneration of the upper motor neurons around the spinal cord, resulting in the sclerosis, or scarring and hardening, of the region (The ALS Association, N.D.). Often times, this process leads to the patient slowly losing muscular function, starting with localized areas, but then gradually spreading throughout the body (The ALS Association, N.D.). One therapeutic approach to curing ALS is find a way to halt the neuromuscular degeneration before it can spread throughout the nervous system, also known as neuroprotective therapy (Rothstein, 1996). The biggest and most widely studied form of neuroprotective therapy involves the use of antioxidants to eliminate reactive oxygen species, as ALS patients have been shown to have high levels of oxidative free radicals compared to those without the disease (Rothstein 1996). It is also known that these very same free radicals have strong neurodegenerative properties (Bukharaeva et al., 2015). Oxidative stress can cause damage to nucleic acids, receptors, and even the membranes themselves (Bukharaeva et al., 2015). Antioxidants change the form of these free radicals into less toxic substances (Rothstein, 1996).

Previous research has been done to find antioxidants that effectively prevent oxidative damage at the neuromuscular junction, and although a perfect mixture has not been found, several noteworthy substances have been identified (Oyewole & Birch-Machin, 2015). Coenzyme Q10 has been shown to be the most effective antioxidant to date (Spindler et al., 2009). It is a specialized antioxidant that works particularly well in ALS patients (Spindler et al., 2009), and it is one of the few antioxidant supplements that are currently able to be used for human consumption (Spindler et al., 2009). Glutathione is another antioxidant that has shown great promise in *in vivo* studies done in FALS transgenic mice (Weiduschat et al., 2014). One commonality between the two is that both directly affect the mitochondria, and that both have unknown mechanisms to their effects (Oyewole & Birch-Machin, 2015). As a result, we hypothesized that both antioxidants may have a summative effect that is presently unknown to science (for example, see Oyewole & Birch-Machin, 2015). Such an effect would suggest that a mixture of both antioxidants could be more effective in clinically treating ALS.

Our study investigated the effect of Coenzyme Q10 and Glutathione, individually and together, on the neuromuscular junction. We hypothesized that Coenzyme Q10 and Glutathione would produce a combined effect on preventing neuromuscular degradation. We predicted that because both antioxidants target the mitochondria and have similar effects, that they will assist one another further in preventing degeneration. To test this assertion, we used a setup for intracellular recordings using the superficial flexor muscles of crayfish (Wyttenbach et al., 1999). We measured the amplitude of EJPs of the crayfish with solutions with Glutathione, Coenzyme Q10, and both Glutathione and Coenzyme Q10 respectively. We found that there was no additive effect of using Glutathione and Coenzyme Q10 together in preventing neuromuscular degeneration.

MATERIALS AND METHODS

Preparation and Electrophysiology

It was important that we chilled the crayfish in an ice bath before beginning to subdue them and prevent movement. This made the dissection easier and reduced discomfort to the crayfish. Additionally, for purposes of the experiment, a bigger crayfish with a bigger tail proved to be more optimal for capturing a nerve. After at least 10 minutes in the ice bath, we grabbed the crayfish from its posterior side to remove it from the bath and used a pair of dissection scissors to cut the entirety of the tail off from the rest of the body. Then the crayfish was placed back in the ice bath.

Solutions

We used different solutions for various purposes in this experiment. The glass microelectrodes contained 3M KCl. We immersed the crayfish in standard crayfish ringer's solution (5.4mM KCl, 196mM NaCl 2.5mM MgCl26H2O, 10mM Hepes Buffer, and 13.5mM CaCl2 2H2O). The Ringer's Solution ensured that the neurons of the superficial extensor muscles remained active throughout our experimentation. Approximately 100mL of Ringer's Solution was sufficient in submerging the crayfish. Our antioxidant solutions were composed of Ringer's solution with either Coenzyme Q10, Glutathione, or both Coenzyme Q10 and Glutathione. Rather than adding hydrogen peroxide directly to the solution, we used a double syringe to replace it another solution, which was the same as the first but with hydrogen peroxide. The hydrogen peroxide had a concentration of 0.33 mM. The antioxidants were made as 1000x stock solutions in dimethylformamide (DMF) for Coenzyme Q10 and water for Glutathione. Glutathione was diluted into Ringer's Solution to a final concentration of 20 uG/mL and Coenzyme Q10 was diluted into Ringer's solution to a final concentration of 10 uG/mL.

Data Collection

We collected data by measuring the amplitude of postsynaptic potentials in the crayfish extensor muscle. These postsynaptic potentials were produced by stimulating a nearby nerve with twin pulses and the delay between the pulses was constant. The change in voltage was read by the microelectrode which was impaled in the muscle fiber, and displayed on a computer. Since twin pulses were used to stimulate the nerve, two EJPs were produced for each recording. This dual stimulation is significant because it allowed for interpretation of the difference between each EJP. In our analysis, we calculated the difference in percentage between the two readings using the formula Percent Change of EJP=[(EJP1)-(EJP2)/(EJP1)]x100. These calculations allowed us to better analyze our data as the raw numbers may vary significantly from cell to cell. We also recorded the amplitudes of each EJP, to determine the overall effect of hydrogen peroxide on the neurons of the neuromuscular junction. We compared the average EJP amplitude, and average percent change between first and second readings of twin pulse stimulation, of each variable to determine our final results.

Data Analysis

Our data was collected over a period of time, and we needed to isolate the pre-treatment data points from the post-treatment data points. We therefore compared twenty data points before the treatment to the last twenty data points recorded after the treatment, to maximize the difference between the two. We also needed to do two comparisons for each trial other than the control. The first compared the EJPs before and after the addition of the antioxidant solution. Although we did not expect there to be any difference, this comparison isolated the possibility of the antioxidants having side effects. The second comparison was between the EJPs recorded after the addition of the antioxidant and the EJPs recorded after the addition of hydrogen peroxide.

RESULTS

The goal of our experiment was to determine the effect of the antioxidants Glutathione and Coenzyme Q10 on the crayfish neuromuscular junction. Specifically, we investigated whether they would be effective in reducing oxidative damage caused by hydrogen peroxide. Previous research identified Glutathione and Coenzyme Q10 as potentially useful antioxidants (Oyewole & Birch-Machin, 2015), and it is possible that they could produce a cumulative effect when mixed together.

In our experiment, we applied these antioxidants to the crayfish extensor muscle and measured EJP amplitudes resulting from twin-pulse stimulation. The EJPs were an indicator of oxidative damage because damaged nerves would produce smaller EJPs. We also measured twin pulse facilitation, since changes to the paired-pulse ratio indicate presynaptic alteration.

Table 1

Treatment	Percent change from antioxidant(%)	T-test for antioxidant(p-value)	Percent change for H2O2(%)	T-test for H2O2 (p-value)
Control	N/A	N/A	-55.42	3.71×10^{-11}
Coenzyme Q10	298.15	1.64×10^{-55}	-48.55	1.16×10^{-27}
Glutathione	2.08	0.38	3.66	0.15
CoQ10 + Glutathione	12.12	5.22×10^{-11}	0.05	0.88

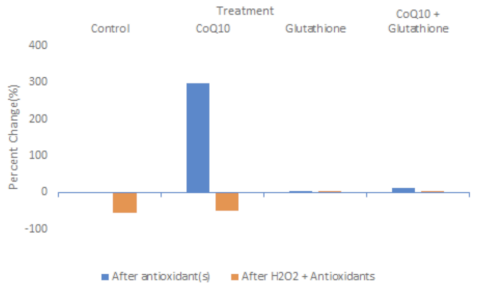

Figure 1. The effect of treatments on amplitude of EJPs. This graph shows the percent change in amplitude of EJPs before and after the addition of the solutions indicated. Each treatment shows the percent change after the addition of the antioxidant, as well as the change after the addition of hydrogen peroxide with antioxidants. T-tests were used to analyze the results. The t-tests compared the data from before and after the treatment to determine if there is a significant difference. The p-values for each test are shown in Table 1.

Table 2

Treatment	Percent change from antioxidant(%)	T-test for antioxidant(p-value)	Percent change for H2O2(%)	T-test for H2O2 (p-value)
Control	N/A	N/A	-195.81	7.28×10^{-11}
Coenzyme Q10	25.85	0.57	30.78	0.39
Glutathione	57.23	0.07	3.66	0.03
CoQ10 + Glutathione	15.90	0.38	-10.60	0.84

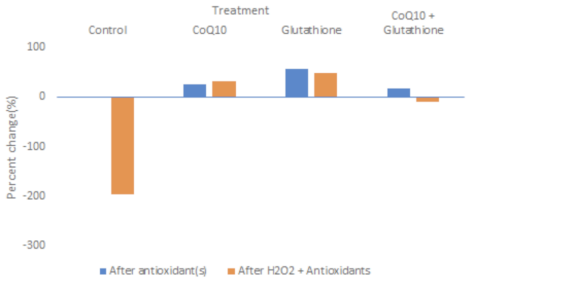

Figure 2. Effect of treatments on twin-pulse facilitation of EJPs. This graph shows the percent change in twin-pulse facilitation of EJPs before and after the addition of the solutions indicated. Each treatment shows the percent change after the addition of the antioxidant, as well as the change after the addition of hydrogen peroxide with antioxidant. T-tests were used to analyze the results. The t-tests compared the data from before and after the treatment to determine if there is a significant difference. The p-values for each test are shown in Table 2.

In our first experiment, we only immersed the crayfish in hydrogen peroxide, so there is no data regarding antioxidants. This group was our control group, and we used it to assess the baseline of oxidative damage. The hydrogen peroxide supported the outcome we expected by causing the most neural degeneration of any group. We found significant decreases in both amplitude and twin-pulse facilitation. Our first experimental group, using only Coenzyme Q10, proved to be problematic. We were unable to hold a single muscle cell for the entire duration of data collection, which caused our amplitude data to be erratic and random. This randomness is because different muscle cells produce EJPs of different average amplitude. Although we tried to take the average of different readings from several different cells to standardize the data, it failed to be reliable nonetheless. Although the p values were quite small (1.64×10^{-55} and 1.16×10^{-27}), they do not verify the amplitude data for Coenzyme Q10 because the data sets were taken from different muscle cells, causing the results to be random. However, the data on twin-pulse facilitation from the Coenzyme Q10 tests was reliable because facilitation is measured in percent change, which does not vary between cells. The p-values returned for other data vary quite a bit and do not seem to indicate a significant difference between any of the antioxidant solutions. For example, the Coenzyme Q10 + Glutathione facilitation t-tests yielded p-values of 0.38 and 0.84 for the antioxidant and H2O2 treatments, respectively. The only clear distinction that can be made is that the control trial, with only hydrogen peroxide, caused significant degeneration of the nerve verified by extremely low p-values (3.71×10^{-11} for amplitude of EJPs and 7.28×10^{-11} for facilitation), and that the addition of antioxidants caused significant reduction of that damage. The antioxidant solutions had higher p-values, indicating that there was not a significant change, and even in the case of lower p-values did not show large neurodegeneration. In fact, it is possible that in the trials with antioxidants – especially Glutathione – synaptic facilitation was actually increased. A possible explanation for this increase is that the antioxidants might have effectively combated both the artificially applied hydrogen peroxide and naturally occurring reactive oxygen species. However, the data is not conclusive enough to confirm this theory.

DISCUSSION

While our results provided some information about the effect of Glutathione and Coenzyme Q10, they were not entirely dependable. It was shown that Glutathione, both individually and when combined with Coenzyme Q10, is effective in preventing oxidative damage. However, the data on the EJP amplitude of Coenzyme Q10 were inconclusive. Furthermore, there did not seem to be an additive effect when the two antioxidants were used

together. It is hard to distinguish whether the mixture was more effective for two reasons. Having missed individual results for Coenzyme Q10 created challenges when comparing our data. Additionally, while both Glutathione and the mixture group produced reliable data, the results suggest that there were no differences between treatments.

Although previous research suggests potential benefits from combinational treatment (Oyewole & Birch-Machin, 2015), there is a lack of knowledge when it comes to the specifics. There are many different antioxidants, and it could be that only certain combinations produce a significantly better effect. Although both Coenzyme Q10 and Glutathione have been observed to function in preventing oxidative damage, there is no clear information on their comparative success (Mari et al., 2009; Spindler et al., 2009). Considering this observation, our study supports prior studies affirming that these antioxidants are effective, but neither supports nor contradicts previous research relating to the additive effect of combining antioxidants.

Future research in this area is essential to discovering new treatments for ALS, and this research should expand in two directions. The most significant area to be studied is other mixtures of antioxidants. There are many untested permutations and combinations of antioxidants that we were unable to explore in our study. For example, Tiron, another supposedly powerful antioxidant, might be added to one of our antioxidants, and produce a different effect (Oyewole & Birch-Machin, 2015). Besides being more creative in trying different antioxidants, future research should also devote more time to precision. Although we were able to retrieve a good amount of reliable data, the majority of it was irrelevant or undependable. A vast quantity of precise data would be needed to truly determine if combining antioxidants has a cumulative effect.

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