

The Effects of C4 Pre-Workout Supplement on Zebrafish Embryos

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Abstract

The purpose of the experiment was to observe and record the side effects on zebrafish embryos when they were exposed to solutions of C4, a pre-workout supplement. Many people use C4 and may not know how it can affect them, in the zebrafish embryos the C4 causes high mortality rate amongst the embryos. In the two trials that were conducted, five different concentrations of C4 were tested, and in each trial, a control was set up, which contained only Embryo Media and no C4. The higher concentrations, tested in the first trial, included 22 mg/ml, 44 mg/ml, and 66 mg/ml of C4. The second trial tested lower concentrations: 0.32 mg/ml and 2.64 mg/ml of C4. The hypothesis was supported in that there was a high mortality rate in the wells that contained concentrations of C4. Additional observations of the living zebrafish embryos revealed that they had a rapid heart rate. The results of the experiment possibly suggest that many of the zebrafish embryos died due to an exponential increase in their heart rate. These effects observed could pose a risk to developing human embryos.

Introduction

With many people trying to achieve specific workout goals, supplements have been used to help achieve these goals faster. C4 is a pre-workout powder that is prepared by mixing the powder, that comes in a miniature package, with water and consuming it; people use C4 to enhance their performance during workouts. The company Cellucor, assures the powder will provide an explosive surge of motivation and will help increase the focus of the user (Cellucor, 2016). Many weightlifters indicate that they detect more enthusiasm in the weight room after using the product, but there has also been multiple deaths and horrid side effects associated with C4, weightlifters restrain from going over the recommended daily intake, which is 2 scoops, in fear of the side effects.

C4 contains beta alanine, creatine nitrate, arginine, caffeine, and an energy blend all which are suppose to give the user a physical, mental, and strength boost. C4 is banned in many sports because of an ingredient that C4 contains, synephrine, which may give athletes an edge over their opponent (Corpus Compendium, 2013). The most alarming thing about C4 pre-workout may be the warning label on the package, which informs the user not to use the product for more than 8 weeks and not to consume any caffeine throughout the day if the supplement is taken, this includes products such as coffee and tea. Furthermore, the label reveals that the product is not intended for people under the age of 18, and the label gives a lengthy list of medical conditions for which the person considering using the product should talk to a physician before use. A research experiment at the National Center of Biotechnology

Information was conducted on how fatigue resistant people are when they use a pre-workout. The total number of leg press repetitions at 75% of one repetition maximum revealed that people using a pre-workout could on average get 2 more repetitions than people not on a pre-workout (Spradley, et al., 2012) This is why people use pre-workouts and protein supplements because it gives them superior results in the gym.

An article written by Steve Roy described the effects that C4 has on the human body. Some of the side effects include nausea and vomiting, tingling of the skin, tunnel vision, heart arrhythmias, and high blood pressure (Roy, 2015). C4 and many workout supplements are not tested by the FDA, so that means that many workout supplements could be unsafe and harm people. The company's selling workout supplements evaluate the safety of their product before selling, so it may be there definition of a safety risk and not the FDA's.

The purpose of the experiment was to observe the effects of C4 on zebrafish embryos, which were used since they are model organisms and develop about the same way humans would. The field of science that this experiment pertains to is toxicology, which is the quantitative and qualitative study of the adverse effects of toxicants on biological organisms. In the experiment the independent variable was the amount of the C4 that was placed into each well in the experiment, and the dependent variable was the zebrafish embryos after they were exposed to the C4. The experiment was conducted using zebrafish embryos from the University of Wisconsin-Milwaukee. The zebrafish embryos were placed into wells, each contains 10 embryos, and put into experimental groups. The hypothesis stated that if the zebrafish embryos were exposed to concentrations of C4, then there would be a higher mortality rate in the groups containing concentrations of C4.

Methods and Materials

The materials required during the experiment include the following; one bottle stock solutions of C4, one beaker for dead embryos, one sharpie, one bottle of Embryo Media Solution, one disposable pipette for transferring zebrafish embryos, one disposable pipette, one plate with wells, one incubator, one depression slide with coverslip, and one dissecting and compound microscope. Furthermore, goggles were used as a safety precaution when handling the C4 to avoid any exposure to the eyes.

On the first day spawning tanks were set up, and brine shrimp were fed. On day two rinsed embryos were obtained from the teacher, and the plate was labeled with our names and class hour, then the concentrations of each well were labeled. In addition, one well plate was filled with 1 ml of Embryo Media Solution, then remaining cells were filled with the C4 stock solutions, the embryos were divided so each well contained 10 embryos and data was recorded on a data sheet. The exact number of live embryos was recorded on the data sheet, and dead embryos were discarded. The embryos were observed under a microscope and observations were recorded. Then the well plates were placed in an incubator overnight. On day three the plate was removed from the incubator, the dead embryos were removed and placed into a waste

beaker. The remaining embryos and hatched fish, were counted and recorded, the C4 stock solutions were removed from each well plate. The C4 stock solutions were replaced with a fresh C4 solution using a clean pipette each time, the plate was then placed under a microscope and observations were recorded, any developmental markers and abnormalities were recorded in the observations. This step was repeated for all C4 concentrations, then 1-2 embryos were placed in a depression slide with a coverslip. The embryos were observed using a microscope and the data was recorded, this step was repeated for all C4 concentrations, and the plate was then returned to the incubator. On day four, day three's work and observations were repeated and the data was recorded. On day five, work and observations from day three were repeated and the data was recorded. All embryos and hatched zebrafish were then placed in a waste container and disposed of appropriately.

GraphPad was used to perform the t-test, which determined if the data collected was statistically significant.

All of the materials were provided by the Wisconsin Inquiry-based Scientist-Teacher Education Partnership (WInSTEP) Program, which is part of the NIH Science Education Partnership Award (SEPA) Program administered by the University of Wisconsin–Milwaukee and the Children's Environmental Health Sciences Core Center.

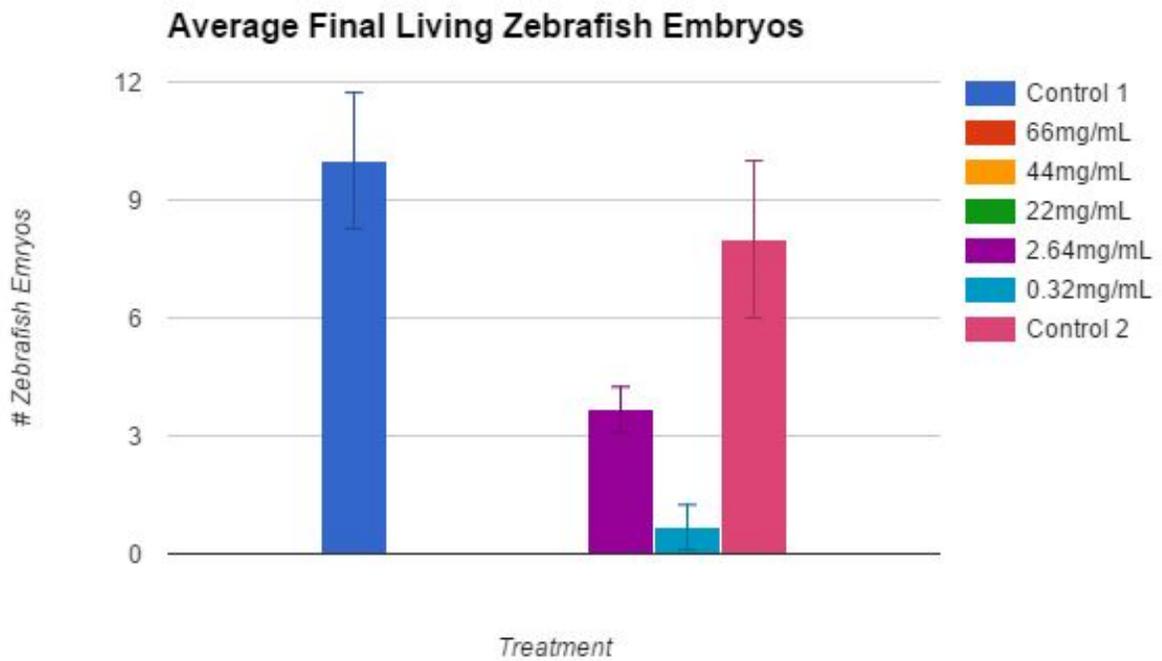
Results

The experiment was done to reveal how workouts supplements like C4 can affect the development of humans. Other experiments like this one can also be used to show how some substances may affect the development of humans. In the experiment the independent variable was the amount of the C4 that was placed into each well in the experiment, and the dependent variable was the zebrafish embryos after they were exposed to the C4. In addition, 2 controls were set up, containing an ocean/embryo solution, and the control was used to compare the effects between the solutions containing C4 and the solutions containing no C4. Furthermore, 2 controls were used because 2 experiments were conducted one containing a larger solution of C4 and the other containing a trace amount of C4. The hypothesis stated that if the zebrafish embryos were exposed to concentrations of C4, then there would be a higher mortality rate in the groups containing concentrations of C4. The hypothesis was supported when there was a mass die off of zebrafish embryos in the C4 solutions, but not in the control. The experiment showed that C4 has a statistically significant effect on zebrafish mortality, which is shown in graph 1 and table 1.

In the experiment the 0.32 mg/ml and the 2.64 mg/ml had the least concentration of C4, and out all concentrations of C4 they had the only surviving embryos at the end of the experiment, which was statistically significant. On the contrary, the 22 mg/ml, 44 mg/ml, and the 66 mg/ml contained the highest C4 concentration and none of the zebrafish embryos were surviving by the end of the experiment, which was statistically significant. Each day observations were made on the zebrafish embryos, and it was observed that the only hatched

zebrafish had a curved spine, which is abnormal and shown in figure 1, so C4 may be a teratogen. In the experiment some of the control zebrafish embryos died, which means a possible error could have occurred that allowed some of the C4 into the control. Furthermore, although it was checked, it cannot be guaranteed that 10 zebrafish embryos were in each well.

Figure 1



The results from the graph 1 reveal that control 1 and 2 contained the greatest amount of live zebrafish embryos compared to the other concentrations that were tested. Furthermore, only two of the concentrations containing C4 had living zebrafish embryos in them by the end of the experiment.

Table 1

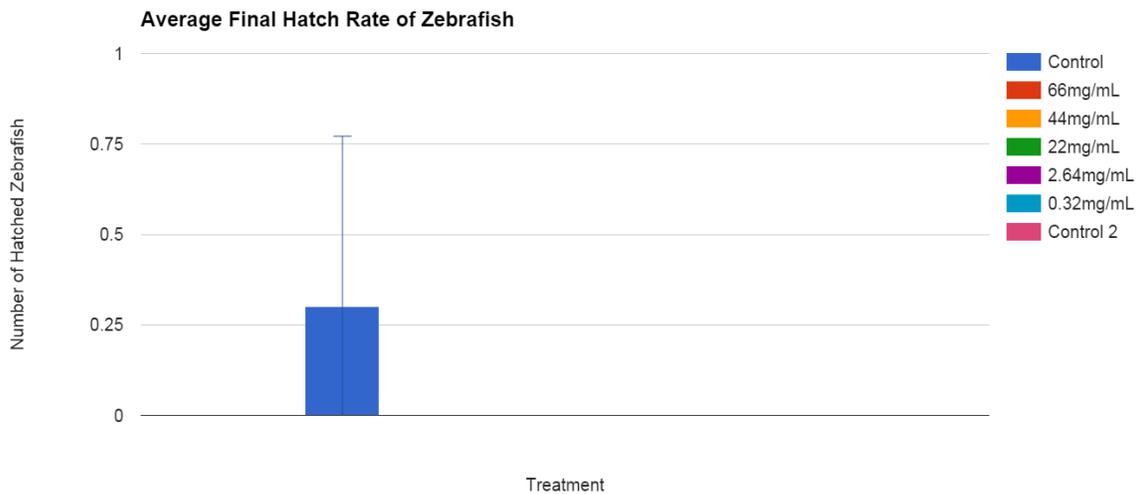
| Treatment | Well 1 | Well 2 | Well 3 | Average | Standard Deviation | Probability | Result |
|-----------|--------|--------|--------|---------|--------------------|-------------|-----------------------|
| Control 1 | 9 | 9 | 12 | 10 | 1.73 | | |
| 66mg/ml | 0 | 0 | 0 | 0 | 0 | p= 0.0006 | Extremely Significant |
| 44mg/ml | 0 | 0 | 0 | 0 | 0 | p= 0.0006 | Extremely Significant |
| 22mg/ml | 0 | 0 | 0 | 0 | 0 | p= 0.0006 | Extremely Significant |
| 2.64mg/ml | 3 | 4 | 4 | 3.67 | 0.57 | p= 0.0226 | Significant |
| 0.32mg/ml | 1 | 0 | 1 | 0.67 | 0.58 | p= 0.0037 | Very Significant |
| Control 2 | 10 | 6 | 8 | 8 | 2 | | |

p > 0.05 (result is not significant)

p < 0.05 (result is significant)

Table 1 shows the number of final living zebrafish, the results taken were deemed significant by using a t-test.

Figure 2



Graph 2 shows the number of hatched zebrafish, in the experiment conducted only one zebrafish hatched, which was in the 0.32 mg/ml. In all other solutions, none hatched, not even in the control.

Table 2

| Treatment | Well 1 | Well 2 | Well 3 | Average | Standard Deviation | Probability | Result |
|-----------|--------|--------|--------|---------|--------------------|-------------|-----------------|
| Control | 0 | 1 | 0 | 0.3 | 0.471407467 | 1 - | - |
| 66mg/ml | 0 | 0 | 0 | 0.0 | 0 | p= 0.3739 | Not Significant |
| 44mg/ml | 0 | 0 | 0 | 0.0 | 0 | p= 0.3739 | Not Significant |
| 22mg/ml | 0 | 0 | 0 | 0.0 | 0 | p= 0.3739 | Not Significant |
| 2.64mg/ml | 0 | 0 | 0 | 0.0 | 0 | - | - |
| 0.32mg/ml | 0 | 0 | 0 | 0.0 | 0 | - | - |
| Control 2 | 0 | 0 | 0 | 0 | 0 | - | - |

$p > 0.05$ (result is not significant)

$p < 0.05$ (result is significant)

Table 2 revealed the same as graph 2, only one zebrafish hatched and the t-test revealed that the results were not significant

Figure 3

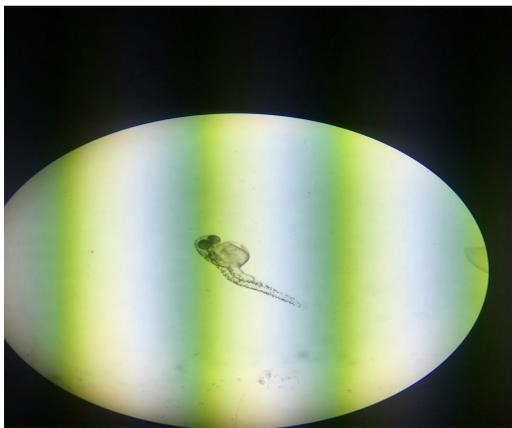


Figure 3 shows a fertilized zebrafish that hatched probably between 48 and 72 hours before the pictures were taken. The structures of the eyes, brain, heart, pectoral fin, somites, and yolk sac look to be intact, but the spine of the zebrafish is curved in an irregular direction.

Figure 4

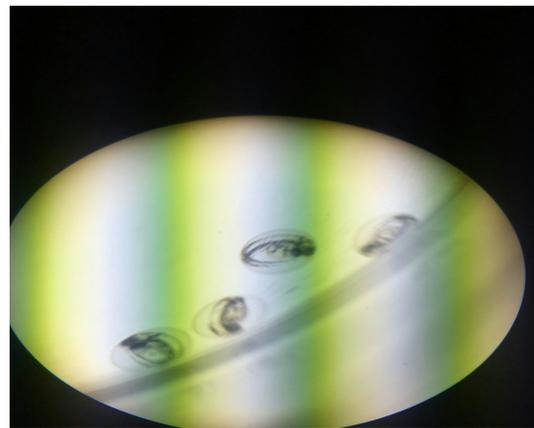


Figure 4 shows fully developed zebrafish embryos inside the egg membrane.

Discussion

Workout supplements have gained extraordinary popularity with people trying to achieve workout goals, so in this experiment C4 a pre-workout, was used to observe how it affects zebrafish embryos. There were 5 solutions that contained C4 in them and 2 controls were used. 2 of the five solutions of C4 contained minimal amounts of C4, and the remaining contained greater amounts. In the beginning of the lab it was hypothesised that if zebrafish embryos were exposed to C4, then there would be a high mortality rate in the groups containing concentrations of C4. This hypothesis was supported by the data collected, in the end of the experiment all of the zebrafish in the high concentration group died and acute effects were observed, and only a few survived in the lower concentration groups. During the experiment only 1 zebrafish hatched, and it was observed that the spine was curved, which is abnormal and shown in figure 3.

Many of the zebrafish in the higher concentrations and lower concentrations of C4 died the next day, it was an exponential decrease in both groups. Also, some of the zebrafish in the control group died, which was peculiar as to why they died if they weren't exposed to anything. The control group had the highest survival rate compared to the groups containing C4, and the lower concentration groups had more surviving zebrafish embryos than the higher concentration groups, which had no living zebrafish embryos. In addition, the experiment produced only one hatched zebrafish, which was found in the 0.32 mg/ml concentration of C4. The exact cause of the massive die off of the zebrafish embryos is unknown. In the deceased embryos there was no movement inside of them and the pigment was darker than the surviving embryos. The surviving embryos had a rapid heart rate, which was observed in the embryos, it was hypothesized that they could have died from the pre longed rapid heart rate that was observed.

There were several errors that occurred during the experiment. In the control there may have been trace amounts of C4, because some of the control embryos died. In addition, each well was suppose to contain 10 embryos each, but even though the embryos were counted it cannot be guaranteed that each well contained 10 embryos.

Future research suggestions include trying even smaller amounts of C4 to observe the effects on the embryos, and may be trying a different model organism to see how it would be affected by workout supplements. In addition, a longer time window for the experiment could be used so that multiple trials can be done and the zebrafish can be observed longer.

The experiment provided results that could further enhance the study of how workout supplements affect humans. The effects reported may coincide with the effects humans may have taking workout supplements. Overall, the results taken revealed that even with small amounts of C4 negative side effects were observed in the zebrafish embryos.

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