The Change of Zebrafish Mortality and Development Caused by Varying Levels of Caffeine Exposure

Erin Coonen
Waukesha North High School

Abstract

Caffeine is known to cause miscarriages and deformities to babies still in the womb. For this study, zebrafish are a model organisms for humans because 70% of protein coding is found in both humans and zebrafish. Also, 84% of genes correlated with human disease has a zebrafish equivalent. This means that zebrafish are similar to humans in their reactions to stimuli. In this study, 40 zebrafish embryos were raised in different levels of caffeine (0.0 mg/mL, 0.05 mg/mL, 0.25 mg/mL and 1.0mg/mL) to determine the effects of this everyday chemical on the development of zebrafish. Previous studies have shown that having too much caffeine can cause a miscarriage. These studies took place over the nine months. This study took place over a week and gave significant results on how caffeine affects the development of zebrafish and possibly humans. The study was conducted to see if zebrafish embryos had development problems if caffeine was introduced into their systems. The zebrafish embryos had many deformities caused by exposure to caffeine and higher level caffeine solutions had more defects than the control or lowest level caffeine solutions. Also, the zebrafish in higher solutions were more likely to die and not hatch. Zebrafish are similar to humans, therefore they were used as a model organism instead of expecting mothers.

Introduction

Caffeine is a chemical that many consume daily but how does this everyday chemical affect the population. Too much caffeine in a day can cause nervousness, insomnia, fast heartbeat, muscle tremors and irritability. Those who don't usually get that much caffeine will feel the side effects more. These have been the effects on a developed person but what about a developing person? The Baby Center (2016) and the March of Dimes (2015) have both done a study on expecting mothers who had different amounts of caffeine. Mothers that had more than 300 mg of caffeine were very likely to suffer a miscarriage (March of Dimes, 2015). This affects the a child and not an adult because the baby was exposed to caffeine for longer than a fully developed person. A baby's metabolism develops while in the womb, which means that the caffeine cannot be processed as quickly as an adult.

The past studies in this field can give insights to how other vertebrates, for example zebrafish, may react to caffeine. The goal of this experiment was to see the relationship between humans and zebrafish and observe any potential effects. That way any expecting women will know the risks and limits of how much caffeine they should consume. The experimental hypothesis was, if zebrafish embryos were exposed to increased levels of caffeine those in the highest levels were to suffer higher mortality and more deformities.

Materials & Methods

The materials used in the experiment were; five course pipettes (one for each stock solution, and one to take out the extra solution in wells), one fine pipette to take dead fish out and water, five 100 mL Beakers (one for each stock solution and a waste beaker), four different stock solutions (a control and the different dilutions of caffeine, 0.05 mg/mL, 0.25 mg/mL and 1.0 mg/mL), a sharpie when writing names and dilutions, 40 fertilized zebrafish embryos (the subject of the experiment), a plate with wells (where the embryos were tested), an incubator at 28.5 °C (helped the embryos developed), dissecting microscope (used to observe the zebrafish) and gloves (a safety precaution throughout the experiment). Most of the materials were supplied 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.

First, a well plate was labeled with names and caffeine concentrations, then gloves were put on after this to protect the researchers from the solutions. Ten embryos were placed into each well and any residual liquid was removed with a fine pipette. 1 mL of the dilutions were placed into each well using a course transfer pipette. The first had 0.0 mg/mL, the next had 0.05 mg/mL, the third well had 0.25 mg/mL and the last a 1.0 mg/mL. After, the embryos were observed under a dissecting microscope which all observations were written down including the number of fertilized eggs, the color of eggs and any movement. The data was collected by observing the color and shape of the zebrafish, for example, a cloudy embryo means that the fish has died. The plate was put into an incubator overnight set at 28.5 °C. After 24 hours, the plate was taken out of the incubator. The zebrafish were observed and data was recorded which included the number of embryos, the number of dead, the number of hatched eggs, the deformities, this was done for each well. These were done by counting the hatched and alive, those that were translucent were alive. The data was then recorded on a piece of paper with pencil and then take pictures of the wells. After the observations, the dead zebrafish were removed and placed into a waste beaker along with the solution from each well, using a fine bore pipette, was replenished by 1 mL of the respective solutions. Then, the plate was put back into the incubator for another 24 hours. Day 2 procedures were repeated for Day 3 and Day 4. Day 5, all the steps were the same as Day 2 but the solution wasn’t refreshed and there was a clean-up. One of the changes was to the recording, which was counting the number of zebrafish with deformities, defined by death, not-hatching, discoloration, deformed spines and fast/irregular heartbeat. Once the cleanup started all the dead fish went into a waste beaker with all the solution possible and the fish in high solution. The fish that were in the control and low solutions were placed in a tank for them to grow. Then all the dated was recorded and chi square analysis was performed to check for statistical significance.

Results

A hypothesis previously stated was that if the zebrafish embryos were exposed to increased levels of caffeine then those in the highest solutions were to suffer higher mortality rates and develop more deformities.

Throughout the experiment the incubator's temperature held at 28.5 °C and the data, with the change of the water each day was in between 11:30 am-1:45 pm. The
time was usually more towards the later of the two numbers. The results were compared to that of the 0.0 mg/mL to allow a conclusion to be reached about the effects of caffeine on the development of zebrafish.

The independent variables for the graphs was time or the type of solution. The dependent variables were the number of zebrafish alive, the number of zebrafish alive and hatched or the number of zebrafish with a defect.

Every 24 hours observations and data were collected. After 24 hours the fish in higher solutions were dead or dying and those in the lesser solutions were a little discolored. After 48 hours, all the fish in the control were hatched and those in highest solution were still in an embryo. In addition, those in the 0.05 mg/mL solution were still an embryo but those in 0.25 mg/mL were either dead or hatched. The third day the differences were more extreme between the control and the caffeinated zebrafish. The controlled were straight and their organs were very visible like the heart, shown in Figure 1. Those in 0.05 mg/mL were hatched but with a curled figure and their organs were visible but lesser than the control. Also, one tried to leave the egg but got stuck and died, shown in Figure 2. The 0.25 mg/mL zebrafish had hatched with the same defects as the 0.05 mg/ml but the curl was tighter and the heart wasn't easily spotted, shown in Figure 3. As for the 1.0 mg/mL, all the zebrafish had died but one, whose embryo was starting to die. For the fourth day, the controlled were thriving and moving casually unlike those in the two middle solutions, the zebrafish were moving erratically, with some having twitches. The highest solution, still had one zebrafish embryo that was barely alive. On the final day, the controlled were still thriving but the 0.05 mg/mL zebrafish, had multiple problems, in that, they had spinal deformities, hearts that were not found as easily as the control. The 0.25 mg/mL had the same problems as 1.0 mg/mL, which was that all had died.

The data was put into several chi squares, they were performed in order to see if the data was caused by the caffeine or if the data was random. Three chi squares were performed in total; the first, was to see if the deaths were caused by caffeine, the second, was used to determine if the hatch rates were caused by caffeine and the third, was used to see if caffeine was the cause of the deformities. For all of the chi squares there was a degree of freedom of 3 and a critical value of 7.82

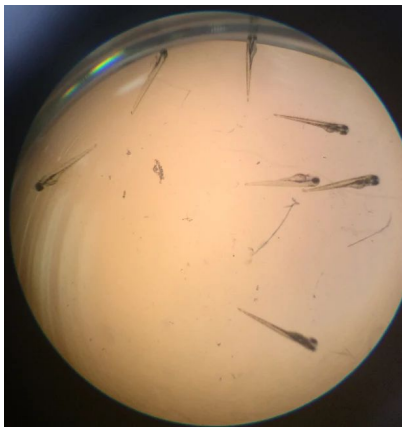

Figure 1: Zebrafish in the control on day 3 without any defects.

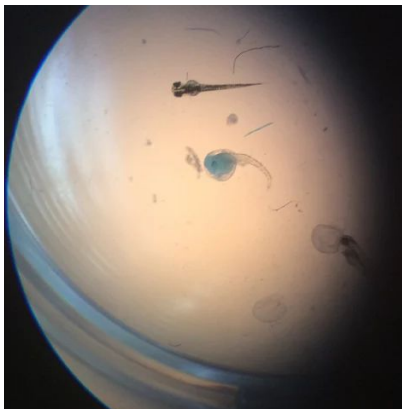

Figure 2: The fish that were exposed to 0.05 mg/ml of caffeine for 72 hours and one had troubles when the fish left the egg.

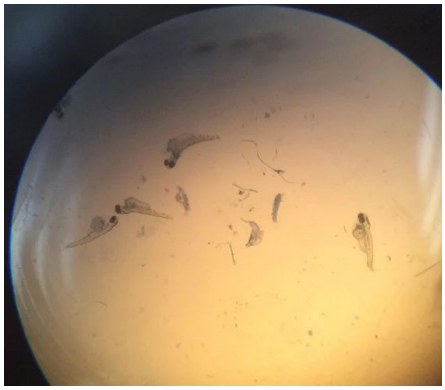

Figure 3: The zebrafish on day three in the 0.25 mg/mL with some spine problems.

This means that at the end of the calculations, if the number is greater than 7.82 than the null hypothesis is rejected.The first was to see whether or not the death rate was related to the solutions. The null hypothesis was that the death rate of a zebrafish was random but after the chi squares were performed, this hypothesis was rejected. The critical value for the chi square was 20.35. The second was used to see if the null hypothesis of the hatch rate being random, like the first, this null hypothesis was rejected. The critical value that was found after the chi square was performed, was 22.50. The third and final chi square was to see if the deformities were random or related to the caffeine. The null hypothesis was that the deformities were random but like the previous chi squares this was then rejected, with a critical value of 26.81.

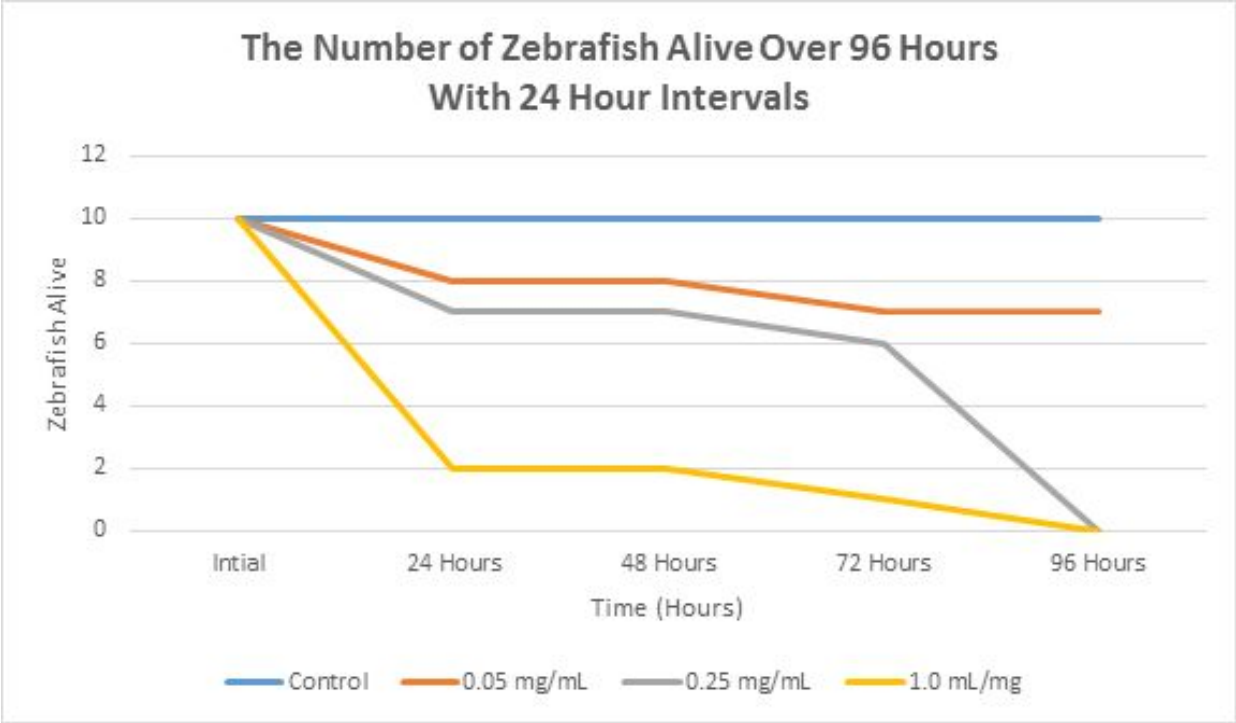

Figure 4- The number of zebra fish alive over 96 hours with 24 hour intervals. This shows the data for how many were alive in each solution. Note the extreme slopes in the high level caffeine solutions and the small slopes in the lowest level caffeine solution.

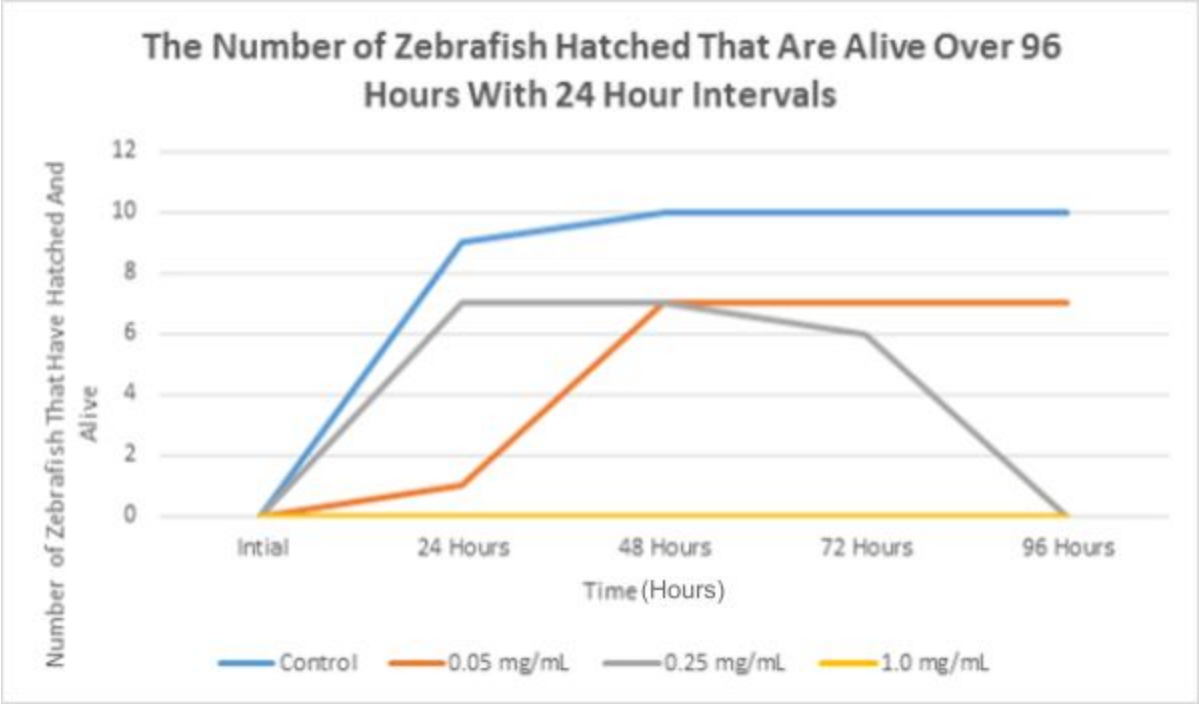

Figure 5- The number of zebrafish hatched that are alive over 96 hours with 24 hour intervals. The data for how many hatched and were still alive. Note that the higher level caffeine solutions had all the embryos die or not hatch.

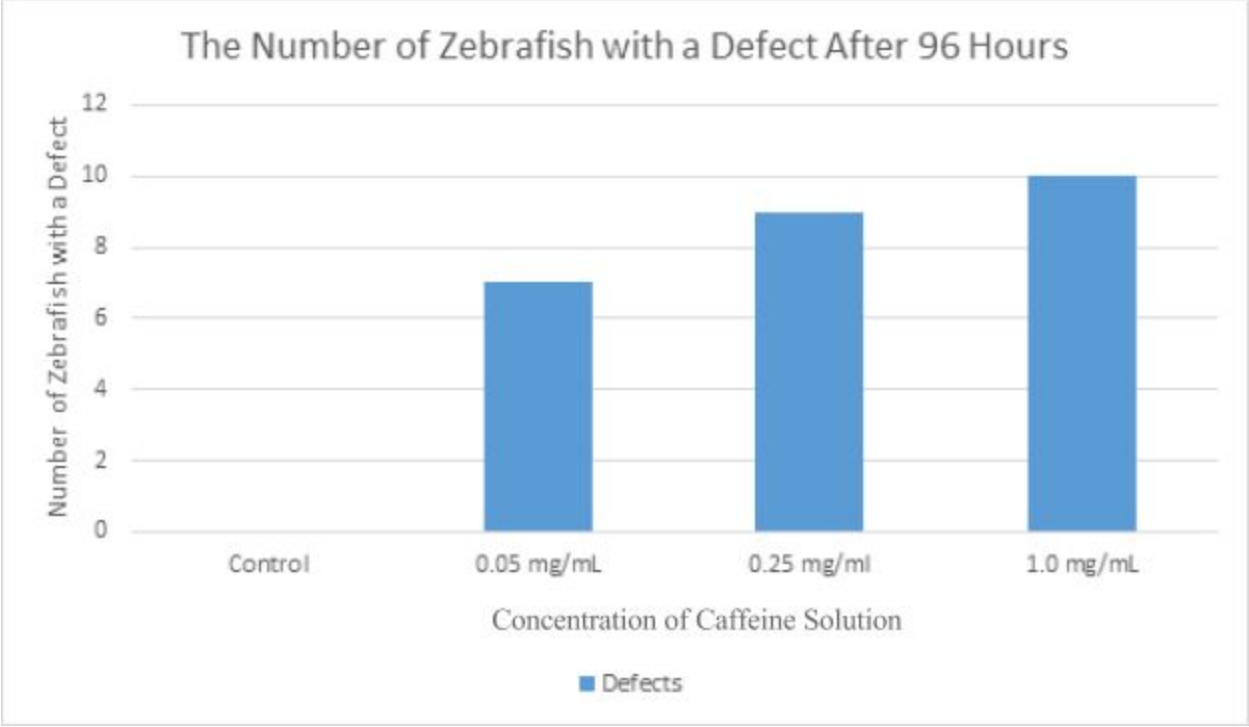

Figure 6- The number of zebrafish with a defect. The data for how many zebrafish had a deformity after 96 hours. A deformity was death, not-hatching, discoloration, deformed spines and fast/irregular heartbeat. Note the increasing levels of deformities showed between caffeine solutions.

Well #	# of Starting Fish	24 Hours Post Fertilization		48 Hours Post Fertilization		72 Hours Post Fertilization		96 Hours Post Fertilization	
		# hatched	# live	# hatched	# live	# hatched	# live	# hatched	# live
1	10	0	10	9	10	10	10	10	10
2	10	0	8	1	7	7	7	7	7
3	10	0	7	7	7	6	6	0	0
4	10	0	2	0	2	0	1	0	0

Table 1- The data table, note the drops in live zebrafish between 72 hours and 96. Also, the drop in live zebrafish in well 4 between the starting and 24 hours.

Discussion

The study on caffeine provided substantial results as to how caffeine affects the development rate and life expectancy. The hypothesis was, if zebrafish embryos were exposed to increased levels of caffeine those in the highest levels were to suffer higher mortality and more deformities. Previous experiments have found that child whose mother had more than 300 mg of caffeine a day were more likely to suffer a miscarriage (March of Dime, 2015).

The chi squares statistical analysis chart was used to determine if the deformities, deaths and hatch rate were caused by caffeine or if the data wasn't connected. Several chi squares were performed, all had a probability greater than the critical value of 7.82. The probabilities for each were: 20.35 for the death rate chi square, 22.50 for the hatched rate and 26.81 for the deformities. This means that caffeine was what caused the deaths, the not hatching and the deformities. Which then supported the hypothesis and similar to the of studies done with caffeine.

As shown in Figure 4 and Table 1, the two highest level of caffeine had all of their fish die. This then, means that if too much caffeine caused the deaths and was then supported by the chi square. This then could translate to expecting mother having a stillborn, miscarriage or the baby to die within a few hours or years.

Each solution had different problems except for the control; the 0.05 mg/mL had twitches or spasms which caused them to act erratically, the 0.25 mg/mL had misshapen spines and spasms. The 1.0 mg/mL were all mostly dead by the second day with none ever hatching.

The miscarriages have been caused by caffeine in other studies and there was one fish that looks as though the zebrafish was a miscarriage. This is just speculation but in Figure 2, the supposed miscarriage looks to have had troubles getting out of the egg and then suffocating. There is no direct answer to how caffeine caused a miscarriage though some hypothesis that the caffeine effects developing cells (Parents, March 2016).

The experiment could have more credibility if the experiment was to be run for a longer time and more times with more zebrafish. More solutions would have also given more accuracy to the data and show how much caffeine is too much. Longer experiment time would then show long term effects as well as the short term effects. Also, if the experiment were to be run more than once, this would then allow a more accurate and have a higher probability of predicting what were to happen in the future. With more fish there would have been a better chance of finding the probability that a fish would survive which was important because this could give percentages to each level of caffeine and then people could plan with how much they would be willing to risk to drink a cup or two. Though the study had limitations many questions are still unanswered, like, how does caffeine cause deformities, is there something in the chemical makeup that reacts with a developing embryos?

To conclude, zebrafish were more likely to die or have deformities in the higher solutions and other studies support this with their own experiments. With too much caffeine deformities, such as death, miscarriages, not hatching and spasms, can occur. There were limitations and ways the experiment could have been improved. Zebrafish are a good model organism for humans and have shown the alarming truth of the chemical consumed everyday.

References

Caffeine during pregnancy | BabyCenter. (2016, December 06). Retrieved from http://www.babycenter.com/caffeine-during-pregnancy

Caffeine in pregnancy. (2015, October). Retrieved from http://www.marchofdimes.org/pregnancy/caffeine-in-pregnancy.aspx#

News Staff. (2013, April 25). Zebrafish Genome Found Strikingly Similar to Humans | Genetics. Retrieved from http://www.sci-news.com/genetics/article01036.html

Gelman, L. (2016, March 24). What Does -- and Doesn't -- Cause Miscarriage. Retrieved from http://www.parents.com/pregnancy/complications/miscarriage/causes-miscarriage/