How Different Concentrations of Caffeine Affect the Mortality Rate of Zebrafish Embryos: Is Drinking Caffeine During Pregnancy a Life or Death Situation?

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Abstract

Caffeine is a controversial drug found in many drinks such as sodas, teas, and coffees. The purpose of this experiment was to test the effects of caffeine on the mortality rate of zebrafish embryos. To execute this experiment, 40 zebrafish embryos were divided and placed into four different solutions containing differing amounts of caffeine. The four solutions were a 0.0 mg/mL or control solution, a 0.05 mg/mL solution, a 0.25 mg/mL solution, and a 1.0 mg/mL solution. Each solution and its embryos were observed over 120 hours. Zebrafish were chosen to be studied for this experiment because of their similarities to humans. For example, zebrafish embryos develop in a similar way that a human fetus develops, so studying them in this experiment gave insights as to how caffeine affects developing human fetuses (Badman, 2000). It was concluded that as the percentage of caffeine in the solution increased, so did the mortality rate of the zebrafish embryos. This data was authorized by research that demonstrated that "large amounts of caffeine have been associated with heart defects in a small number of babies…. lower birth weight and an elevated risk of spontaneous abortion" (Howell, 2005). Other research made it known that when tested on mice, caffeine showed harmful effects and long-term consequences for those mice (Underwood, 2013).

Introduction

Whether caffeine is advantageous or deleterious to the human body, the verdict is still out. However, speculations are currently being made about whether or not caffeine has adverse effects on human fetuses and developing babies. One thing that is commonly known about caffeine is that it speeds up brain activity and gives its consumer a copious amount of energy. “One of the ways caffeine speeds up brain activity in adults is by blocking the activity of a neurotransmitter called adenosine, which acts as a brake on neuronal firing and makes us sleepy” (Underwood, 2013). It is known how caffeine can affect adults, but how can it affect fetuses and developing babies? The Psychiatry Department at Emory University (2005) stated that caffeine does indeed affect developing human fetuses and babies. “During pregnancy, the fetus receives all of its oxygen and nutrients from the mother through the placenta. Caffeine crosses the placenta easily, because of its low molecular weight and high lipid solubility” (Howell, 2005). Large amounts of caffeine have been known to cause adverse effects in fetuses and forming babies. These effects include: heart defects, lower birth weights than is healthful, a higher risk of having a miscarriage, higher heart rates, and behavioral effects such as more startles and tremors, and a lower ability to be consoled. Also, the United States Food and Drug Administration declared caffeine unfit for women who are pregnant and strongly advised the avoidance of caffeine during pregnancy in 1980 (Howell, 2005).

This information indicates that consuming caffeine during pregnancy is not necessarily safe nor is it beneficial. However, more experimentation is needed to determine if the correlation between caffeine and negative effects in fetuses and babies is accurate. Zebrafish are great specimens in which to test this correlation because zebrafish, like humans, are vertebrates. This means that

they are related to humans and may have biological traits resembling those of humans. “Although zebrafish and humans are obviously very different, their embryonic development is remarkably similar…. This comparison extends even to the molecular level--where similar genes perform similar functions in many different species” (Badman, 2000). A hypothesis was formed based on information regarding correlation between caffeine and humans, and the research that indicates the similarities between humans and zebrafish. It was hypothesized that if zebrafish embryos are exposed to an environment containing caffeine, then they will have a lower survival rate than zebrafish embryos that aren't exposed to an environment containing caffeine. The results of the experiment designed around this hypothesis could give insights as to how caffeine affects human fetuses and forming babies.

Materials and Methods

Materials

The solutions used in this experiment include: one premade 0.0 mg/mL solution of caffeine (Instant Ocean Control), one premade 0.05 mg/mL solution of caffeine, one premade 0.25 mg/mL solution of caffeine, and one premade 1.0 mg/mL solution of caffeine. These solutions require a well plate with at least four wells to hold them and 40 zebrafish embryos to be placed in them (ten in each well). In order to remove and replace what is necessary throughout the experiment, one 50 mL beaker, five 1.5 mm pipettes, four 1 mL fine-tipped pipettes, and five pairs of latex-free powder-free vinyl gloves are needed. Additionally, one incubator set to 28.5 degrees celsius is necessary for the storage of the well plate when observations for the day end. For labeling and organization purposes, two strips of chemical labeling tape and one fine tip Sharpie permanent marker are needed. Finally, for observation and documentation purposes, one Bausch and Lomb dissecting microscope and one photographic device (for this experiment, an iPad was used) are needed.

Methods

On the first day of the experiment, 40 rinsed zebrafish embryos were obtained along with the well plate, two strips of chemical labeling tape, and a Sharpie fine tip permanent marker. The zebrafish embryos were divided up into groups of 10 and placed into the top four wells using the 1.5 mm pipette. 1 mL of the premade 0.0 mg/mL caffeine solution was added to the first well (the well in the top left corner), and 1 mL of the premade 0.05 mg/mL caffeine solution was added to the second well. Next, 1 mL of the premade 0.25 mg/mL caffeine solution was added to the third well, and 1 mL of the premade 1.0 mg/mL caffeine solution was added to the fourth well (the well in the top right corner). After that, the cover was placed on top of the well plate, and the two strips of chemical labeling tape were placed on the cover underneath the top wells (which contain the embryos). The first strip of chemical labeling tape was labeled with each solution concentration (0.0 mg/mL was written underneath the first well, 0.05 mg/mL underneath the second well, 0.25 mg/mL underneath the third well, and 1.0 mg/mL underneath the fourth well) using the Sharpie. The second strip of chemical labeling tape, which was placed under the

first one on the well plate cover, was labeled with the student's name, hour of the class, and "Caffeine." Finally, the number of live embryos in both the control (the premade 0.0 mg/mL solution), and the experimental (the premade 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL solutions) wells were recorded on the on the student data sheet. The well plate was then given to the teacher to be placed in the Incubator. All of the premade solutions, along with their designated 1.5 mm pipettes, went into a refrigerator after being used.

On the second, third, and fourth day of the experiment, the well plate was obtained from the teacher and placed under the Bausch and Lomb dissecting microscope for observations. First, the date and time of the observations were recorded. Next, extensive data about each well's embryos were recorded on the student data sheet. The number of embryos present, hatched, alive, and dead in each well were recorded. After that, a detailed description of the movement and color/shade of the embryos were recorded for each well. In addition, a photo of one or more live embryos in each well was taken using the iPad provided by the school (four photos in all, meaning one photo of each well). Each photo was taken through the Bausch and Lomb dissecting microscope ocular lenses while the microscope was at its highest setting (25 times ocular lens). After all of the pictures were taken and labeled with their correct day and well numbers, latex-free powder-free vinyl gloves were put on the hands and one 1 mL fine-tipped pipette was used to suck out all of the dead embryos from the first well (0.0 mg/mL premade caffeine solution). All of the dead embryos were placed in the 50 mL beaker along with any excess solution that came with them. Next, the premade 0.0 mg/mL caffeine solution was sucked out of the well and placed into the same 50 mL beaker as the dead embryos, leaving only the live embryos in the first well. The well was then refilled with 1 mL of the 0.0 mg/mL caffeine solution using the 1.5 mm pipette designated for that premade solution. The process of sucking out the dead embryos and replacing the correct solutions in each well was repeated for the other three wells. After all of the dead embryos (the appearance of which is shown in Figure 1) were sucked out and the solutions were replaced, one final check under the microscope was made to make sure that all of the live embryos were in each well. The cover was then placed back on the well plate. Finally, the well plate was given back to the teacher to be placed in the Incubator, the 1 mL fine-tipped pipette was disposed of, and the beaker with the old solutions and dead embryos was rinsed out in the sink and left on the table to dry.

The same process as on Day 2-4 was repeated on Day 5, except for the end of the process. Instead of the well plate going back into the incubator, the living fish were taken out and placed into the tank provided. All of the solutions were then disposed of down the sink drain provided, and the well plate was rinsed out. The well plate was then thoroughly cleansed and dried, thus concluding the observation portion of the experiment.

Safety Precautions

Latex-free powder-free vinyl gloves must always be worn when sucking anything out of or putting any premade solutions into any of the wells. When sucking out the dead embryos from the wells, make sure that only the dead embryos are sucked out, not any live ones. The process of sucking out the dead embryos and replacing the solutions must be completed in order from the control (the 0.0 mg/mL solution) to the solution with the highest concentration of caffeine (the 1.0 mg/mL solution), so as to not contaminate any solutions. Along with that, make sure not to mix up any of the 1.5 mm pipettes so as to avoid contamination issues.

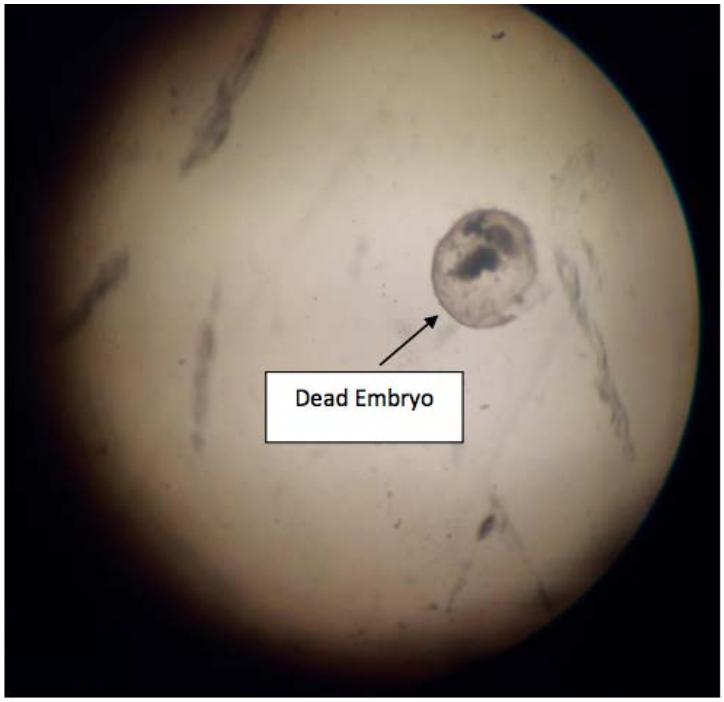

Figure 1: A dead embryo is shown to help enhance the understanding of what a dead embryo looks like and help improve the distinction between live and dead embryos.

Results

It can be hypothesized that if zebrafish embryos are exposed to an environment containing caffeine, then they will have a lower survival rate than zebrafish embryos that are not exposed to an environment containing caffeine. The results of this experiment based upon this hypothesis provided insights as to how caffeine affects zebrafish embryos during development. One of the most notable insights gathered during the experiment was the variation in the mortality rate of zebrafish embryos and developing zebrafish who are exposed to caffeine. In turn, this research could help grow the understanding of the effects of caffeine on human fetuses and forming babies. This is because a human's embryonic development is exorbitantly similar to that of a zebrafish (Badman, 2000). The independent variables in this experiment were the four solutions (the 0.0 mg/mL or control solution, the 0.05 mg/mL solution, the 0.25 mg/mL solution, and the

1.0 mg/mL solution). The dependent variables in this experiment were the zebrafish embryos. The controls for this experiment were the amount of eggs in each well, the type of eggs, the size of the wells, and the temperature of the incubator. The dependent variable was affected by the independent variable because as the concentration of caffeine increased, so did the mortality rate of the zebrafish embryos, depicted in figure 2.

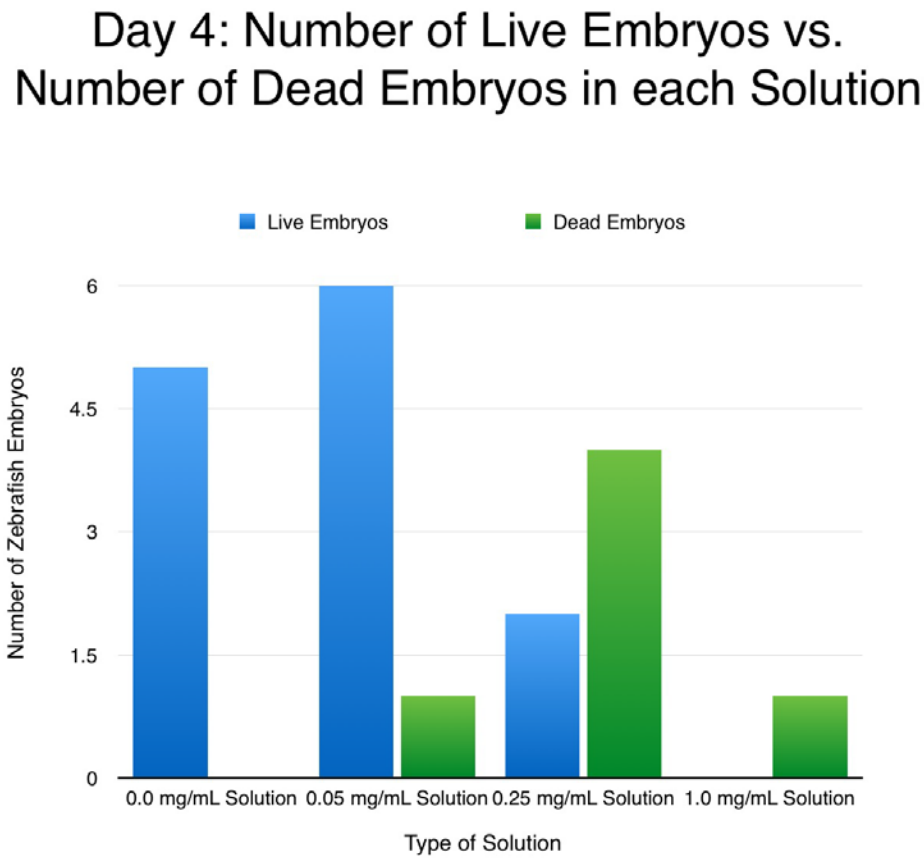

Figure 2: This bar graph illustrates the amount of live (blue) and dead (green) zebrafish embryos observed on Day 4 of the experiment. As the percentage of caffeine in the solution increases, the amount of dead embryos observed also increases, and the amount of live embryos decreases.

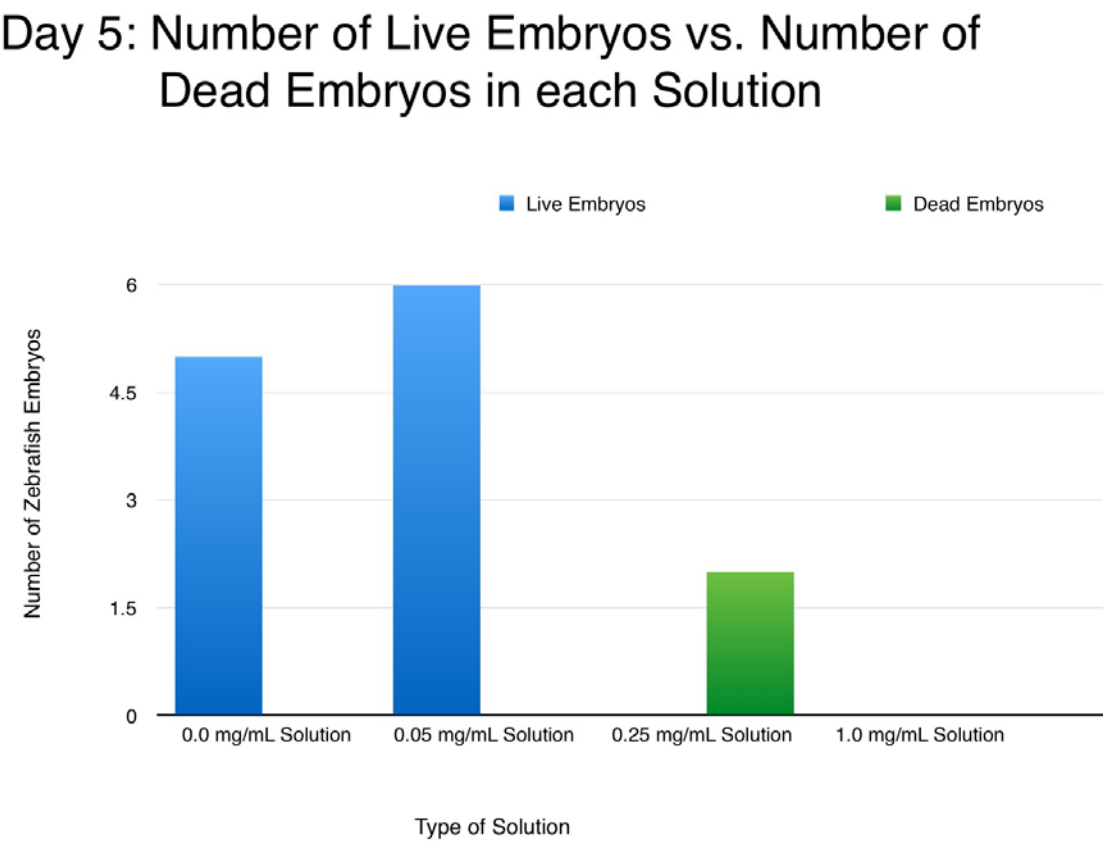

Figure 3: This bar graph illustrates the amount of live (blue) and dead (green) zebrafish embryos observed on Day 5 of the experiment. As the percentage of caffeine in the solution increases, the amount of dead embryos observed also increases.

Both Figure 2 and Figure 3 show that, overall, the amount of living embryos decreased as the percentage of caffeine in the solutions increased. When Figure 2 and Figure 3 were compared, the results showed that in the 0.0 mg/mL solution and the 0.05 mg/mL solution, the amount of live embryos stayed consistent. This comparison also showed that the amount of live embryos decreased in the latter two solutions (the 0.25 mg/mL solution and the 1.0 mg/mL solution).

Chi-Square Analysis Chart Day 5

Live

Treatment	O	E	(O-E)	(O-E)^2	(O-E)^2/E
Control (0.0 mg/mL)	5	4.23	5-4.23	0.77^2	0.5929/4.23 = 1.4
0.05 mg/mL	6	5.0769	6-5.0769	0.9231^2	0.852/5.0769 = 0.1678
0.25 mg/mL	0	1.69	0-1.69	-1.69^2	2.8561/1.69 = 1.69
1.0 mg/mL	0	0	0-0	0^2	0/0 = 0

X^2 = 3.2578

Dead

Treatment	O	E	(O-E)	(O-E)^2	(O-E)^2/E
Control (0.0 mg/mL)	0	0.769	0-0.769	-0.769^2	0.591361/0.769 = 0.769
0.05 mg/mL	0	0.923	0-0.923	-0.923^2	0.851929/0.923 = 0.923
0.25 mg/mL	2	0.31	2-0.31	1.69^2	2.8561/0.31 = 9.213
1.0 mg/mL	0	0	0-0	0^2	0/0 = 0

X^2 = 10.905

Table 1 X^2 + Table 2 X^2 = 14.1628

O = Number of Zebrafish Observed
E = Expected Number of Zebrafish (according to the expected value formula)

Figure 4: These charts are an accurate representation of how the chi-square statistic was calculated on Day 5 of the experiment using the chi-square formula.

Following the completion of the experiment, a chi-square analysis was completed with the results of each day. Day 5 of the chi-square analysis proved to be the most significant because it was able to give results as to how the different solutions affected the mortality rate of the zebrafish overtime. The chi-square analysis for Day 1 of the experiment was insignificant because, at that time, no zebrafish in any solutions had died, so the mortality rate could not yet be measured.

The null hypothesis for this experiment was rejected because the chi-square analysis revealed a score of 14.1628 for the comparison between live and dead zebrafish embryos on Day 5 of the

experiment, as shown in Figure 4. Since the critical value of the chi-square analysis is 7.82, and the score previously noted is greater than that, the null hypothesis was indisputably rejected. This means that the experiment results were not due to chance alone. Also, the null hypothesis for this experiment was rejected because after all of the day's scores were calculated, it was rejected 60% of the time, which was the majority of the days.

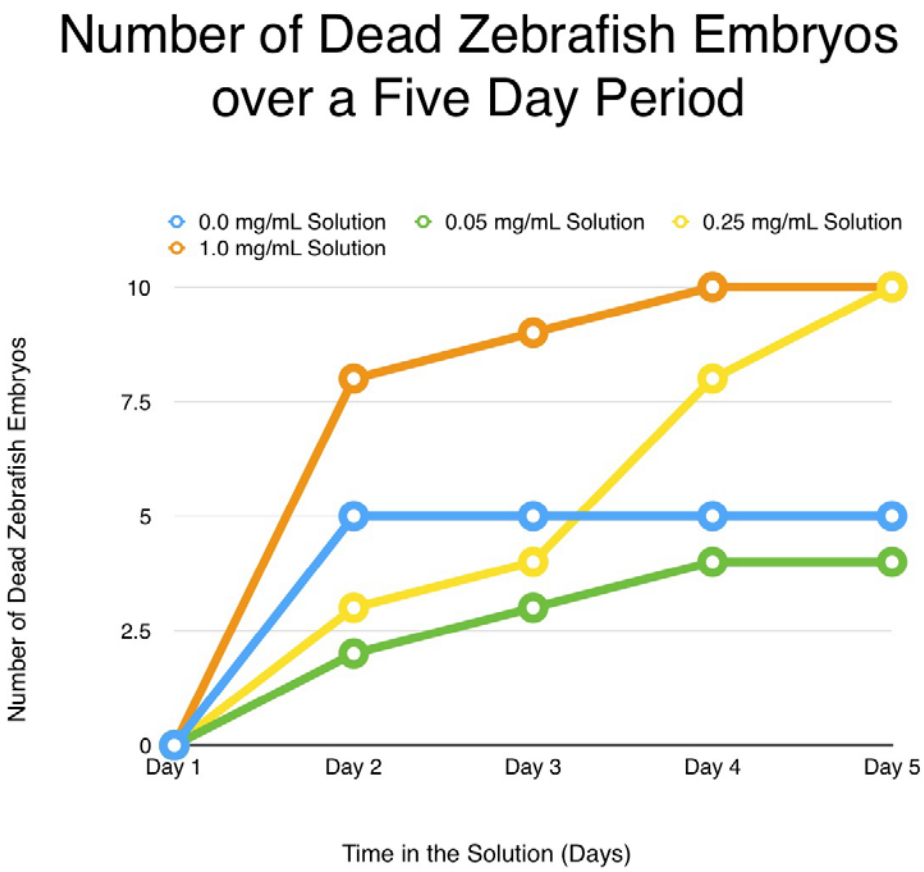

Figure 5: This line graph depicts the amount of dead embryos over the course of the experiment.

As shown in Figure 5, in general, the two solutions with the higher amounts of caffeine resulted in higher mortality rates than the solutions with the lower amounts of caffeine. On Day 5 of the experiment, this graph showed that in both the 1.0 mg/mL solution and the 0.25 mg/mL solution, there was a 100% mortality rate of the zebrafish.

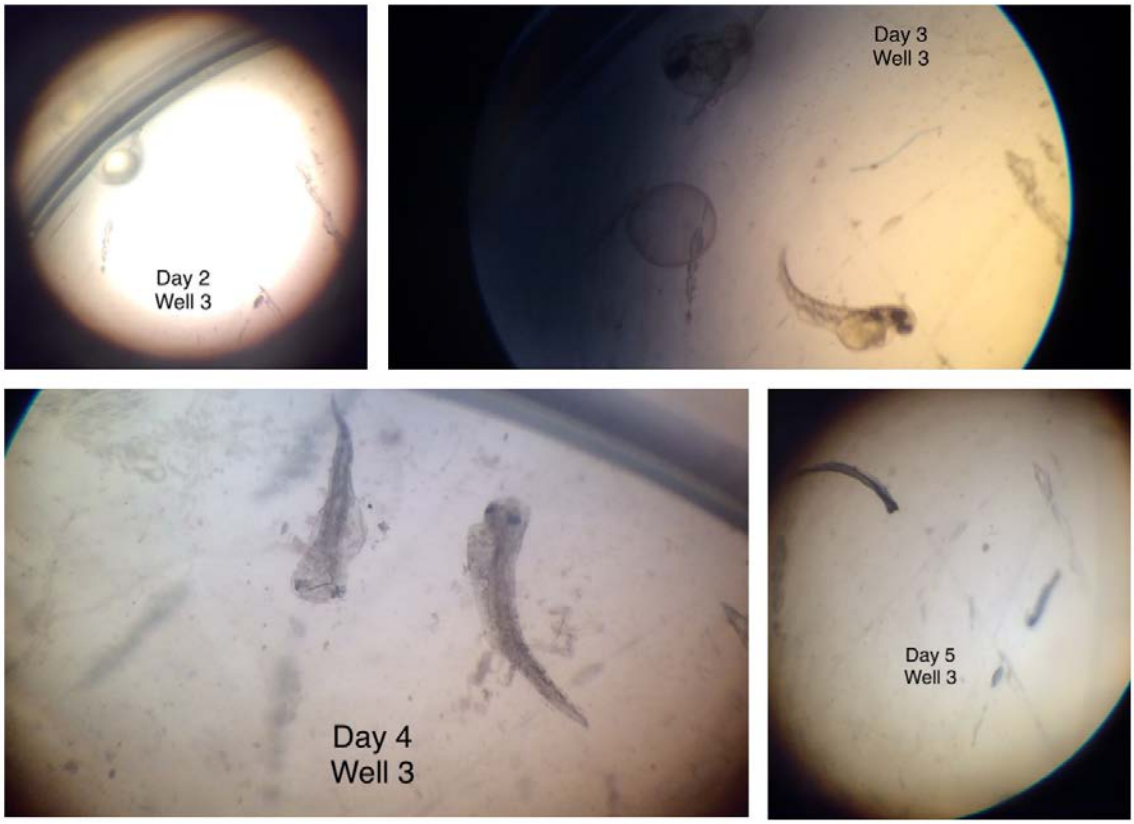

Figure 6: These photos depict the increasing decline of the zebrafish embryos in the 0.25 mg/mL solution. The top left photo depicts the embryos observed on Day 2 of the experiment, and the top right photo portrays a hatched zebrafish embryo on Day 3 of the experiment. Next, the bottom left photo illustrates two hatched zebrafish embryos who were considered alive, but were nearly dead. They showed no signs of movement or heartbeats on Day 4. Then, on Day 5 of the experiment, the bottom right photo depicts the dead zebrafish (located in the top left corner of the photo) observed.

Figure 6 clearly shows that the 0.25 mg/mL solution caused major physical changes to the zebrafish embryos in it. The bottom left photo shows a color change in the zebrafish from a healthy transparent yellow to a sickly gray. Also, detrimental deformities to the zebrafish can be seen in the bottom left photo, including loss of shape and unhealthy eye movement across the head. Drastic changes were seen on Day 4 and Day 5 of the experiment in the 0.25 mg/mL solution, which ultimately resulted in the death of all ten of the zebrafish embryos in the solution.

Discussion

The results of this experiment provide valuable information that confirms the initial hypothesis. The hypothesis stated that if zebrafish embryos are exposed to an environment containing caffeine, then they will have a lower survival rate than zebrafish embryos that aren't exposed to

an environment containing caffeine. This hypothesis was established based on research that found that “large amounts of caffeine have been associated with heart defects in a very small number of babies. Large amounts of caffeine have also been related to lower birth weight and an elevated risk of spontaneous abortion” (Howell, 2005). The purpose of this experiment was to further the claims about caffeine to determine their possible effects on the mortality rate of zebrafish. A meager amount of research was found on deaths among fetuses and infants exposed to caffeine, “but a new study in mice offers the controversial suggestion that at larger doses, caffeine can impair memory and increase the risk of having seizures” (Underwood, 2013). In addition, Dr. Carla Silvia, a neuroscientist at University of Coimbra in Portugal, stated, about the caffeine experiment on mice previously mentioned above, that “‘caffeine exposure in early life resulted in long-term consequences’ for the mice” (Underwood, 2013). Those long-term consequences were studied in this experiment, but on zebrafish embryos instead of mice.

The data from the conducted experiment validated the hypothesis that if zebrafish embryos are exposed to an environment containing caffeine, then they will have a lower survival rate than zebrafish embryos that aren't exposed to an environment containing caffeine. This can be proven by looking at Figure 2 and Figure 3. Figure 2 shows that, on Day 4 of the experiment, there were no deaths in the solution without the presence of caffeine (the 0.0 mg/mL solution); it instead showed that there was a high amount of deaths in the 0.25 mg/mL caffeine solution. By Day 4 of the experiment (72 hours post fertilization) two-thirds of the observed zebrafish embryos in the 0.25 mg/mL solution were dead, and 100% of the total zebrafish embryos in the 1.0 mg/mL solution were dead; this makes it very perspicuous that caffeine does indeed have a substantial effect on the mortality rate of zebrafish embryos. Also, Figure 3 shows that there is a definite effect of caffeine on the mortality rate of zebrafish embryos because precisely 0% of the embryos in the 0.25 mg/mL and 1.0 mg/mL solutions survived; it also shows that the two solutions with the lowest amounts of caffeine (the 0.0 mg/mL and 0.05 mg/mL solutions) had between 50% and 60% of their embryos survive. This data proves that caffeine does affect the mortality rate of zebrafish embryos because the embryos in the 0.0 mg/mL solution had no deaths after the 24 hour observation, while the amount of deaths of the embryos in the other solutions gradually increased overtime. An intriguing piece of evidence found during the course of the experiment was that half of the zebrafish embryos in the 0.0 mg/mL (Instant Ocean Control) solution were dead after 24 hours.

A speculation was formed that this evidence is abnormal because no other zebrafish from the 0.0 mg/mL (Instant Ocean Control) solution were found dead after the 24 hour observation, as shown in Figure 5. A possible error in this experiment could have been that, at the beginning of the experiment, previously dead zebrafish embryos could have been placed into the 0.0 mg/mL (Instant Ocean Control) solution. This is a rational speculation because at the end of the experiment, more zebrafish embryos were dead in the 0.0 mg/mL (Instant Ocean Control) solution than in the 0.05 mg/mL solution (as can be verified in Figure 5); this should not have happened because research revealed that even a little caffeine is more deleterious than no

caffeine. “A small amount of caffeine does get into breast milk, so limit caffeine if you're breastfeeding. Breastfed babies of women who drink more than 2 to 3 cup of coffee day may become fussy or have trouble sleeping” (March of Dimes, 2015). Although zebrafish embryos are not breastfed, this research still helps to verify that caffeine, even in diminutive amounts, can have adverse effects on developing zebrafish. Therefore, there was no logical reason as to why there would be more zebrafish embryos dead in the 0.0 mg/mL (Instant Ocean Control) solution than in the 0.05 mg/mL solution. Also, it’s justifiable to speculate that the 0.0 mg/mL (Instant Ocean Control) solution had no effect on the zebrafish embryos because after the initial death of five of the embryos, no other zebrafish died in the 0.0 mg/mL (Instant Ocean Control) solution; ergo, something must have gone wrong in order to cause five of the zebrafish embryos in the 0.0 mg/mL solution to die.

Although the effects of caffeine on the mortality rate of zebrafish embryos have been tested, how caffeine affects the development, hatch rate, and movement patterns of zebrafish embryos is still unknown. However, as shown in Figure 6, it was very apparent to see that, on Day 4 of the experiment, the two zebrafish alive in the 0.25 mg/mL solution were quite deformed, as proven by their color and lack of shape. Despite this data, more research and experimentation is needed to be able to show significant results as to how caffeine affects the deformities of developing zebrafish. One way that this experiment could be enhanced by observing and making note of not only the mortality rates of the zebrafish, but also of the movements, deformities, and/or hatch rates of the zebrafish. Another way that this experiment could be improved is by making sure that all embryos are living when they are put into their wells, so as to not besmirch the data about the mortality rates of the zebrafish by having dead embryos prior to the experiment.

It was learned from this experiment that caffeine does indeed affect the mortality rate of zebrafish embryos. This can be proven by Figure 4 because the null hypothesis was rejected for Day 5 of the experiment, which means that the results of the experiment were not due to chance alone. This is significant because it shows that the solutions of caffeine do indeed have an impact on the mortality rate of the zebrafish embryos. Since caffeine was the substance tested on the zebrafish embryos, and because the null hypothesis for this experiment was rejected, that signifies that caffeine does affect the zebrafish. With that, Figure 4 analyzed the mortality rate of the zebrafish embryos, so this experiment proved that caffeine specifically affects the zebrafish embryos’ ability to live. The data from this experiment was used to make the claim that, because humans and zebrafish have similar a embryonic development (Badman, 2000), caffeine also has adverse effects on humans. This was authenticated by research that states that, “During pregnancy, the fetus receives all of its oxygen and nutrients from the mother through the placenta. Caffeine crosses the placenta easily, because of its low molecular weight and high lipid solubility” (Howell, 2005). This information communicates that caffeine does indeed reach the fetus, so it’s plausible to speculate that it affects fetuses, too.

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