The Effect of Caffeine on Zebrafish Embryo Development

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Abstract:

Zebrafish are very commonly used in experimentation to model the effects of certain toxicants on human development since there are many similarities between zebrafish embryos and human fetuses. The goal of this experiment was to find out how caffeine affects the development of zebrafish. In this experiment zebrafish are exposed to 0.05 mg/mL, 0.25 mg/mL, and 1 mg/mL of caffeine, while also being compared to a control in an instant ocean solution. At 24, 48, 72, and 96 hours post fertilization, observations were made on how many zebrafish survived and how each fish is developing. Most of the zebrafish that were exposed to caffeine showed malformations. As the caffeine concentration increases, the disfigurations seem to get more prominent. These abnormalities included bent tails, curved spines, cardiac edemas, and yolk sac edemas. This was very evident in the 1 mg/mL solution as well as a 100% mortality rate by 96 hours post fertilization. This is important because many people consume caffeine everyday, and this will reflect how caffeine will affect human development. This experiment shows that caffeine intake should be limited especially with pregnant women in order for the infant to be healthy. If this is not monitored, there is a possibility for the infant to develop abnormally or even result in a miscarriage.

Introduction:

Zebrafish have become popular models when researching the effects of certain environmental factors during development. These fish have a similar physiology and neuroanatomy to that of humans and many other mammals during the stages of development (Kaleuff et al. 2013). This makes these embryos good comparisons when trying to find the effects of different substances, whether that be physical or behavioral. A major benefit to using zebrafish for experimenting is how quickly development takes place, which can be easily observed as the eggs are transparent. The embryos are also produced in large numbers, making it possible to test hundreds at a time, increasing the accuracy of the results (Kumar et al., 2012). Finally, zebrafish are inexpensive, making them very affordable for these experiments (Kaleuff et al., 2013).

According to the Alcohol and Drug Foundation, Caffeine is the most common stimulant drug in the world whether it be in the form of coffee, energy drinks, tea, or chocolate. While many people consume it, not many are aware of the negative effects caused by it (ADF, 2018). If an overdose occurs there is a possibility of irregular heart rate, nausea, vomiting, arrhythmia, and even death (Rana et al., 2010). It is advised that pregnant women should avoid caffeine as much as possible because the caffeine crosses through the placenta. Babies can not metabolize caffeine well and it will also change the baby's sleep patterns. Studies of animals have shown that caffeine can cause birth defects, premature labor, and miscarriage (Caffeine Intake During Pregnancy, 2018). How does caffeine impact the development of zebrafish embryos? With all of this past research on the effects of caffeine, it can be clearly hypothesized that if zebrafish embryos are exposed to caffeine, then the survival rate will decrease and the fish will experience possible deformities and heart problems.

Materials and Equipment:

- Each Solution of Caffeine (0.05, 0.25, and 1 mg/mL)
- 1 bottle of Instant Ocean/ Embryo Media Solution
- Methylene Blue solution
- Waste Container
- Sharpie
- Tape
- Disposable pipettes (minimum bore, 1.5 for transferring eggs to the observation container and manipulating them in the container)
- Disposable pipette, 1 mL
- Plate with wells
- 28.5℃ Incubator
- Depression slide with cover slip
- Dissecting and compound microscope
- Zebrafish embryos

Procedure:

Day 1:

1. Obtain the zebrafish embryos from the teacher.
2. Label the plate with name and class hour. Label the control and caffeine concentrations of 0.05 mg/mL, 0.25 mg/mL, and 1 mg/mL, in each row of the plate using the Sharpie provided.
3. Fill the 4 wells in the control row with 1 mL of Instant Ocean/Embryo Media solution using the disposable pipette. Fill the remaining wells with the appropriate caffeine solutions. Divide the embryos so there are 10 embryos in each well. Label the plate on the student data sheet.
4. Record exact numbers of live embryos on the student data sheet. Dead embryos should be discarded.
5. Observe the embryos under the dissecting microscope. Record observations on the student data sheet.
6. Place each plate in the 28.5℃ incubator overnight.

Day 2:

1. Remove plate from the incubator.
2. Remove dead embryos from plate using the disposable pipette. Squirt all of the dead embryos into the waste beaker. Be careful to only remove dead embryos.
3. Count remaining embryos, hatched fish, and record in data table.
4. Remove the 0.05 mg/mL caffeine solution from each well of the plate by tilting the plate so the embryos settle and remove the liquid from the top.
5. Replace the 0.05 mg/mL solution with the appropriate fresh caffeine stock solution using a clean pipette.

6. Place the plate under dissecting microscope and record observations on student data sheet. Note/describe any developmental markers and abnormalities. Repeat for all caffeine concentrations.
7. Remove 1-2 embryos and place on the depression slide with cover slip. Observe the embryos using the compound microscope. Record observations on student data sheet. Repeat for all caffeine concentrations.
8. Return the embryos to the well in the plate.
9. Return the plate to the appropriate 28.5℃ incubator.

Day 3:

1. Repeat Day 2 work and observations. Record all data.

Day 4:

1. Repeat Day 2 work and observations. Record all data.

Day 5:

1. Repeat Day 2 work and observations. Record all data.
2. Place all embryos and fish in waste container. The teacher will properly dispose of the organisms.

Above Procedure was taken from: SEPA Program- UW- Milwaukee

Data:

Data Table 1: Number of Living Embryos Over Time

Number of Living Embryos					
Concentration	# of starting fish	# alive 24 hpf	# alive 48 hpf	# alive 72 hpf	# alive 96 hpf
Control	40	34	28	26	25
0.05 mg/mL	40	33	30	26	22
0.25 mg/mL	40	36	35	35	32
1 mg/mL	40	39	39	15	0

Data Table 1 shows how many zebrafish were living for each concentration when starting and at 24 hpf, 48 hpf, 72 hpf, and 96 hpf.

Figure 1: Survival Rate of Embryos Over Time

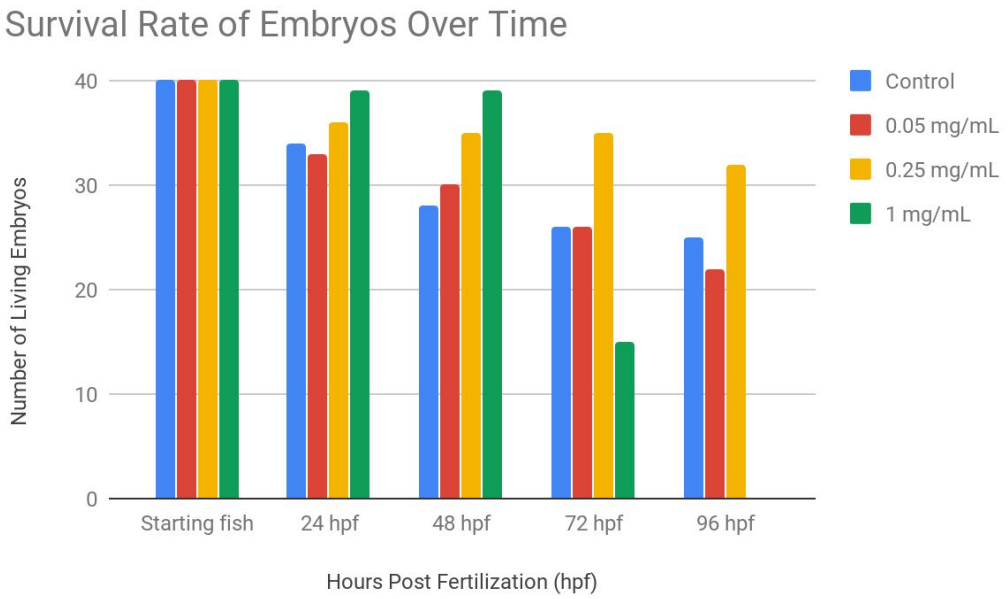

Figure 1 shows the number of zebrafish that survived at 24 hpf, 48 hf, 72 hpf, and 96 hpf for the fish in the instant solution, and the concentrations of caffeine at 0.05 mg/mL, 0.25 mg/mL, and 1 mg/mL.

Data Table 2: Survival Rate Comparisons

Caffeine- Survival Rate		
Comparison	P-Value	Significance
Control vs .05 mg/mL- 24 hpf living	0.8345	Not Significant
Control vs. 0.25 mg/mL- 24 hpf living	0.5847	Not Significant
Control vs. 1 mg/mL- 24 hpf living	0.2148	Not Significant
Control vs. 0.05 mg/mL- 48 hpf living	0.8005	Not Significant
Control vs. 0.25 mg/mL- 48 hpf living	0.3677	Not Significant
Control vs. 1 mg/mL- 48 hpf living	0.1768	Not Significant
Control vs. 0.05 mg/mL- 72 hpf living	1.0000	Not Significant
Control vs. 0.25 mg/mL- 72 hpf living	0.2731	Not Significant
Control vs. 1 mg/mL- 72 hpf living	0.324	Not Significant
Control vs. 0.05 mg/mL- 96 hpf living	0.7141	Not Significant
Control vs. 0.25 mg/mL- 96 hpf living	0.3125	Not Significant
Control vs. 1 mg/mL- 96 hpf living	0.0118	Significant

Data Table 2 shows the comparison of the survival rates between the control and each concentration of caffeine for 24 hpf, 48 hpf, 72 hpf, and 96 hpf by using t- tests.

Figure 2: Control

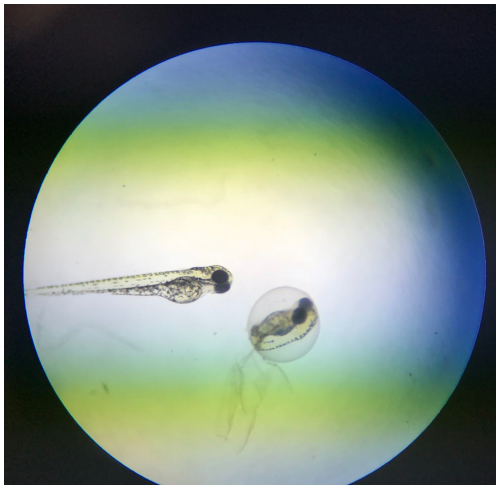

Figure 2 shows a zebrafish from the control group developing normally after 72 hours post fertilization.

Figure 3: 0.05 mg/mL

Figure 3 shows a zebrafish from the 0.05 mg/mL caffeine solution after 72 hpf. It has a curved spine.

Figure 4: 0.25 mg/mL

Figure 4 shows two embryos at 72 hpf in 0.25 mg/mL caffeine solution. They both have bent tails and have both a yolk sac edema and cardiac edema.

Figure 4: 1 mg/mL

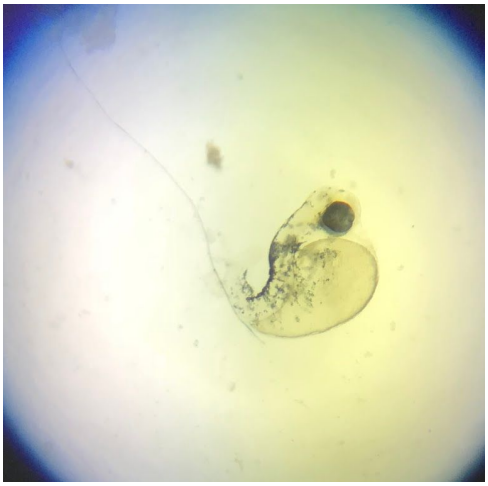

Figure 5 shows a zebrafish in the 1 mg/mL caffeine at 72 hpf. It has a large yolk sac edema as well as a deformed spine.

Data Analysis:

A t-test was used with a p-value set at 0.05 to test statistical significance. An unpaired t-test is used to determine if two groups have different average values. This analysis allows predictions to be made from the data set. In the data set, the survival rate of zebrafish in the control was being compared to the caffeine concentrations of 0.05, 0.25, and 1 mg/mL. There was only significance shown at 96 hours post fertilization for the control vs. 1 mg/mL of the caffeine solution. The reason for this was the drastic change in the survival rate in this caffeine solution where all of the fish died within the last day whereas the control was stable. All of the others were not significant, due to the fact that in the earlier stages of development, there were more disfigurations than deaths in each solution, and the mortality rates were in very close range with the control solution. At 72 hpf for the control vs. 0.05 mg/mL there was a P-Value of 1.0000 because they both had an equal amount of zebrafish alive within each solution.

Results:

This experiment was designed to track the effects that different caffeine concentrations have on zebrafish. The development of these fish were observed and compared to those in the control, where normal development was seen. The death rate of these fish were also observed and recorded at 24 hpf, 48 hpf, 72 hpf, and 96 hpf. The independent variables in this experiment were the 0.05, 0.25, and 1 mg/mL caffeine solutions as well as the instant ocean solution. The dependent variables were the death rates in each solution and the different abnormalities that resulted. The different solutions affected these dependent variables. The control was the fish found in the the instant ocean solution, as the other zebrafish in the caffeine solutions were being compared to these ones. Each day the fish in the control were always seen to be developing normally, unlike those in the caffeine solutions. As the concentration increased, more deformities were seen in the fish. This included cardiac edemas, yolk sac edemas, bent tails, and curved spines. These were all more prominent in the 0.25 mg/mL and the 1 mg/mL or as the amount of caffeine increased. Unlike the abnormalities there was not a strong pattern when looking at the death rate. In the 0.05 mg/mL and the 0.25 mg/mL the survival rate was always consistent with the control. The same thing was observed with the fish in the 1 mg/mL until 72 hpf where the survival rate was nearly cut in half, and at 96 hpf where none of the zebrafish survived. This experiment made it very clear that caffeine has a major effect on the development of zebrafish.

Discussion:

A large amount of data was taken from this experiment. While there was not much significance for survival rate other than at 96 hpf for the control vs. 1 mg/mL, it is evident that caffeine plays a role in the survival of a fetus, due to the fact that all of the zebrafish in the solution with high levels of caffeine died by the end of the experiment. This partially supported the hypothesis that if zebrafish were exposed to caffeine, then the survival rate would decrease. While the fish in the lower levels of caffeine stayed fairly consistent with that of the control solution, the survival rates most definitely decreased with the 1 mg/mL solution. In other experiments it has been found that depending on the dosage of caffeine, the heart rate will decrease tremendously, and eventually stopping completely(Rana et al., 2010). This might

have been what happened to the zebrafish in this concentration. There were many significant qualitative observations found that supported the second part of the hypothesis that caffeine would cause abnormalities in the development of zebrafish. It was clear that as the caffeine concentration increased, the disfigurations in the fish got significantly worse. In the 0.05 mg/mL many of the fish had curved spines, whereas when examined the zebrafish in 0.25 mg/mL, almost all of them had cardiac edemas as well as yolk sac edemas. Some had bent tails which resulted in them only ables to swim in a circle. Then there was the 1 mg/mL where it was seen that there was a delayed hatching of the fish. Once it reached 72 hpf, at first glance it could have been assumed that none were hatched, when in reality the spines of the zebrafish were so deformed and the yolk sacs so enlarged that the fish looked like it was still in an egg. There can be many limitations in all experiments. It would have been very helpful if there was better equipment available to find the heart rate of the zebrafish. That way, it would have been easier to tell if the heart rate was slower or faster than those in the control group. Another limitation could have been the amount of zebrafish that was used in the experiment. If more could have been tested on, the results would have been more accurate. Overall, this experiment strongly suggests that high doses of caffeine will only have negative effects on developing zebrafish, reflecting in that of human fetuses. The data collected leads to the conclusion that pregnant women should avoid consuming high levels of caffeine, as it might not only cause malformations in the developing infant, but could cause a miscarriage as well. This is supported by many sources saying that pregnancies that result in a miscarriage showed a higher caffeine intake than those with live births(Greenwood DC et al., 2010).

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