The Effects of Caffeine on the Mortality Rates and Development of Zebrafish Embryos

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Abstract:

Zebrafish are important organisms when wanting to know how some substances may affect human development. They are commonly used in a laboratory setting. In this experiment zebrafish were exposed to different levels of caffeine to observe resulting effects. Caffeine is so commonly consumed, it is not considered a toxin by the general population. It is known to cause development issues and even death in growing fetuses, so by conducting this experiment, it is possible to come to a conclusion on the effects of caffeine and the safety of human health. For this investigation 160 zebrafish were exposed to 3 different caffeine levels of 0.05, 0.25, and 1 mg/mL, all compared to one control solution of instant ocean. Data was collected every 24 hours for a total of 96 hours. In regards of mortality, there was one significant result in the highest concentration of caffeine when compared to the control solution. All of the zebrafish died in that solution supporting the conclusions of the possible dangers when consuming high levels of caffeine. Throughout the experiment, it was observed that as the caffeine level increases, more malformations develop that become more severe. This shows how important it is that all people should be limiting consumption of caffeine in order to live a healthier life.

Introduction:

Over time, scientists have found zebrafish as a useful resource when it comes to testing different toxins or substances. Zebrafish, as a developing embryo, have a strong similarity to the human embryo in both physiology and neuroanatomy (Kalueff et al., 2013). This allows scientists to come to conclusions of how human embryos may react in different conditions. Zebrafish also breed in large quantities often and are very inexpensive compared to other mammals that may be tested. In addition to that, zebrafish develop quickly and the embryos are transparent allowing scientists to monitor physical changes or abnormalities that may occur (Kumar et al., 2012). The other benefit of having embryos to test on is that they will not be in utero, which would make it difficult to observe early development, and it will not affect the mother as a result like mammals would (Kinth et al., 2013).

Caffeine is a commonly consumed substance throughout the world found in tea, coffee, chocolate, and many energy drinks. According to the Food and Drug Administration (FDA), about 80% of adults from the United States consume caffeine everyday with the recommended safe amount to consume is about 400 mg. This stimulant can also be found in some medications or prescriptions by doctors, but it is linked to increased blood pressure, mental alertness, and can lead to an upset stomach or heartburn (Rana et al., 2010). There are many other health problems connected to the consumption of too much caffeine like insomnia, dehydration, headaches, and abnormal heart rhythm (Caffeine, 2018). There still is not a

considerable amount of research done on the effects of caffeine in the cardiovascular system, and this could be included in children, adolescents, pregnant women, and their fetuses. Pregnant women are not recommended to drink more than 200 mg of caffeine each day as caffeine will enter the bloodstream and can be passed on to the fetus through the placental barrier causing the heart rate or metabolism to increase. Caffeine has been shown to cause birth defects, miscarriages, and fertility problems (Morgan, 2013). Based on some of the known causes, how will different amounts of caffeine affect the development of the zebrafish? It can be hypothesized that different concentrations of caffeine will cause irregular heart rates, malformations, and even death of developing zebrafish embryos.

Materials and Equipment:

- 1 bottle of Instant Ocean/ Embryo Media
- 1 bottle for each stock solution of Caffeine (0.05 mg/mL, 0.25 mg/mL, 1 mg/mL)
- Methylene Blue
- 1 beaker for dead embryos and liquid disposable
- 1 disposable pipette plus extras (1.5 mm for transferring eggs to observation container and manipulating the eggs in container)
- 1 disposable pipette, 1 mL
- 1 plate with wells
- 3x4 well plate with a cover
- 28.5°C incubator
- Sharpie and tape
- 1 dissecting microscope
- Depression slide with cover slip
- Zebrafish embryos

Procedure:

Day 1

1. Obtain embryos from the teacher.
2. Label the plate with name and class hour. Label the control, caffeine concentrations of 0.05 mg/mL, 0.25 mg/mL, 1 mg/mL in each row of the plate using sharpie provided.
3. Fill each well plate in the control row with 1 mL of Instant Ocean/Embryo Media solution using disposable pipette. Fill the remaining wells with 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 the dead embryos into the waste beaker. Be careful to only remove the dead embryos.
3. Count remaining embryos, hatched fish, and record in data table.
4. Remove 0.05 mg/mL caffeine solution from each well plate. Note: Tilt the plate so the embryos settle and remove the liquid from the top.
5. Replace the 0.05 mg/mL caffeine solution with fresh caffeine stock solution using a clean pipette.
6. Place plate under dissecting microscope and record observations on student data sheet. Note: describe any developmental markers and abnormalities. Repeat steps 4 and 5 with 0.25 mg/mL and 1 mg/mL caffeine solutions.
7. Remove 1-2 embryos and place on the depression slide with cover slip. Observe the embryos using the compound microscope. Record observations on the student data sheet. Repeat for all caffeine solutions.
8. Return the embryos to their 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.
2. See Day 4 if experiment is not continued

Day 4

1. Repeat Day 3 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: Embryos Alive After Exposure to Caffeine Concentrations

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 the number of embryos living in each concentration after 24 hpf, 48 hpf, 72 hpf, and 96 hpf.

Figure 1: Survival Rate of Embryos Over Time

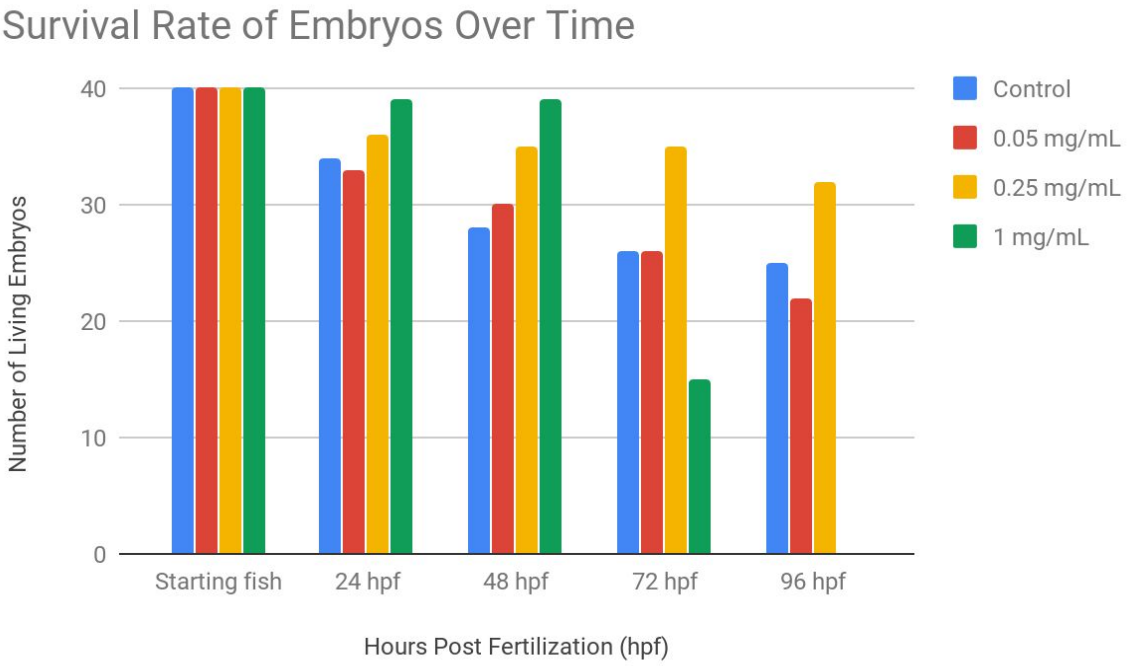

Figure 1 compares the number of living embryos at the start of the experiment, 24 hpf, 48 hpf, 72 hpf, and 96 hpf in each concentration of caffeine.

Data Table 2: Unpaired T-test and Statistical Significance Between Groups

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 how significant the gathered data was with the relationship between the control and each concentration at 24 hpf, 48 hpf, 72 hpf, and 96 hpf using an unpaired t-test.

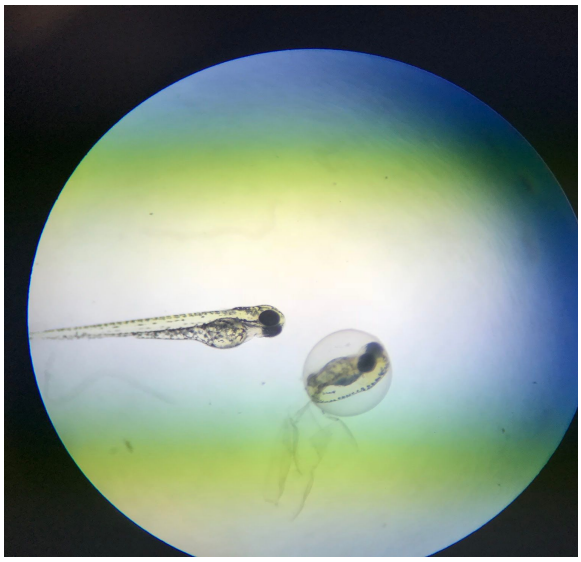

Figure 2: Two zebrafish in the control at 72 hpf developing normally, one still unhatched.

Figure 3: One of the zebrafish in .05 mg/mL at 72 hpf, developed with a curved spine.

Figure 4: Two zebrafish in the .25 mg/mL

Figure 5: A zebrafish in the 1 mg/mL

concentration at 72 hpf, both with cardiac edemas, a yolk sac edema, and bent tails and spines.

concentration at 72 hpf with a yolk sac edema, cardiac edema, and a deformed spine.

Data Analysis:

The statistical test that was used was an unpaired t-test. 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. There was only one significant result with the control vs 1 mg/mL caffeine concentration at 96 hpf. This is because of the sudden change in the death rate within the 1 mg/mL wells. There was very little significance when comparing other results of the experiment. Due to the deaths in the control within 0 and 48 hpf and the fact that there were more malformations in the lower concentrations of caffeine rather than deaths, there was not a lot of significance when comparing the number of zebrafish still alive. An example of this similarity in the number of deaths that took place can be seen when comparing the control and the .05 mg/mL solution at 72 hpf. Because the number of zebrafish alive was exactly the same, there was no significance, leaving the p-value at exactly 1.0000.

Results:

Caffeine is a commonly consumed substance, and it is important to understand how it may affect a developing embryo. In this experiment, zebrafish were exposed to three different concentrations of caffeine at 0.05, 0.25, and 1 mg/mL and were compared to zebrafish developing in the control of instant ocean. The number of deaths were recorded at 24, 48, 72, and 96 hpf. The independent variables in this experiment were 0.05, 0.25, and 1 mg/mL caffeine solutions the zebrafish developed in. The dependent variable was the survival rate of the zebrafish within each solution. The controls in the experiment were the zebrafish that were in the instant ocean. The dependent variable of mortality rate is affected by the difference in the caffeine solutions and can be compared to what is normal by the control. All of the zebrafish in the instant ocean solution developed normally. In the 0.05 mg/mL solution, the only abnormality that was observed in some of the zebrafish was a curved tail causing them to swim in circles, otherwise they developed healthy like the fish in the instant ocean. The zebrafish in the 0.25 mg/mL solution had cardiac edemas, yolk sac edemas, and curved tails. Then in the 1 mg/mL solution, the zebrafish, like in the 0.25 solution had cardiac edemas, yolk sac edemas, and curved tails and spines. At 96 hpf however, all of the zebrafish died in this solution. It also caused a delayed hatching for the first 48 hours. Throughout all of these solutions there was not a large difference in the survival rates apart from the highest concentration, but there was clearly a high number of abnormalities as the amount of caffeine increased. The aim of the experiment was to find the significance that caffeine can have on zebrafish development and how it affects the survival rate. In this experiment it showed that caffeine can have a significant effect on development of zebrafish within high concentrations and can even be fatal. As the concentration of caffeine increases, the number of deformities increases along with the severity

of the deformities. In the highest concentration however, it lead to a large number of deformities and also an increased mortality rate.

Discussion:

The hypothesis at the beginning of this experiment stated that when exposed to different levels of caffeine, there will be change in heart rate, malformations, and death of a developing embryo. After obtaining the results and observations throughout this experiment it becomes clear that there is a significant change in development with many malformations, supporting that part of the hypothesis. The quantitative results that showed the overall mortality rate, however, only showed significance when comparing the control and the highest concentration at 96 hpf, so there was not a clear correlation between mortality rate and caffeine concentration. These results do not strongly support the hypothesis on the aspect of survival rate. With the significant data then, perhaps it is only at the highest concentration that the malformations were so bad, that it eventually caused the heart to stop. In other experiments it was found that as caffeine concentration increased, heart rate decreases, eventually resulting in complete heart cessation (Rana et. al., 2010). This may be the reason that the zebrafish in the 1 mg/mL concentration of caffeine had died suddenly on the last day of the experiment. Through qualitative observations, it was apparent that as the caffeine concentration increased, the deformities became worse. This was consistent within each well. They become progressively worse starting with curved tails in the lowest concentration, enlarged yolk sacs, hearts, and curved spines in the 0.25 mg/mL concentration, and similarly, but in worse condition, cardiac and yolk sac edemas, and deformed spines in the highest concentration. In humans, it is also seen that with the consumption of caffeine, it can cause miscarriages and malformations in fetuses. Safety of the fetuses, like zebrafish, decreases with high caffeine consumption (Morgan et. al., 2013). Some limitations in this experiment include the fact that it is difficult to measure heart rate without proper equipment, so it was not possible in this setting to gain quantitative results to find if it does or does not support that part of the hypothesis. Errors that occurred in this experiment were mainly in the control. When changing the water, some of the embryos accidently got sucked up through the pipette. This resulted in their death causing inaccurate results in the mortality rates. If the experiment was conducted again with better precision and by being more careful, it may show more significant results. The data that was collected throughout this experiment however, leads to the conclusion that any person, but more importantly women who are pregnant, should limit caffeine consumption. This is to ensure that no malformations develop as the fetus grows and so there is not a possible miscarriage from drinking high amounts of caffeine. This claim is supported by many other conclusions from research, stating that caffeine can have many negative effects on human anatomy in growth and development.

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