The Effects of Caffeine on the Mortality Rates and Abnormalities In Zebrafish Embryos

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Abstract

Most people would not consider caffeine to be a toxin, because it is consumed so frequently by the general population. However, exposure to this toxin can have negative effects on any human, and is even more hazardous to pregnant women because of the effects it can have on the unborn child. Zebrafish were used as models to investigate and report findings on these effects. This study exposed 120 zebrafish embryos to 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL caffeine solutions at different hours post fertilization, with the inclusion of 30 embryos that lived in a toxin free environment of Instant Ocean solution, which were used as a control to compare to the experimental groups. All of the embryos exposed to caffeine had abnormalities. The higher the concentration of caffeine the more abnormalities were present, and the higher the mortality rate was. The embryos exposed to 1.0 mg/mL caffeine solution all had extreme abnormalities and almost a 100% mortality rate by 96 hours post fertilization. Caffeine is very common in human diets. This experiment is important because it shows that caffeine usage should be cautioned and limited. These findings can lead people to limit their caffeine intake and live a healthier lifestyle.

Introduction

According to the FDA, about 90% of the world's population consumes caffeine, making it one of the world's most used drugs (Pietrangelo, 2014). About 80% of adults in the U.S. consume around 200 mg of caffeine everyday, both in foods and medicines (Medicines In My Home, 2007). Numerous studies with animals have shown that caffeine can cause many reproductive problems, including birth defects, premature labor, and reduced fertility. However, there have been no conclusive studies with humans. Because of this, and other conflicting studies, the March of Dimes suggests that women should not consume more than 200 mg of caffeine per day (Caffeine Intake, n.d.). Once pregnant, many women reduce caffeine consumption, yet it is estimated that 70-80% of pregnant women still consume caffeine (Pastore, et. al., 1995). When consumed, caffeine can travel through the bloodstream and into the placenta, which can cause the baby's heart rate and metabolism to increase. With large consumption, caffeine can even slow fetal growth and increase the chance of miscarriage (Pietrangelo, 2014). There are also other risks. The Mayo Clinic states that consuming excess caffeine can lead to insomnia, restlessness, nervousness, increased heart rate and even muscle tremors. *Medical News Today* reported that intake of caffeine while pregnant may increase the chance of a low birth weight baby and early death, and the majority of doctors say that from infancy to adolescence people should avoid caffeine consumption (Whiteman, 2015). The zebrafish has many traits that allow it to be a very important model in the research of vertebrate development, specifically concerning humans. Zebrafish have many genomic and molecular similarities to humans, making many of the discoveries found in zebrafish applicable to humans (Veldman, et. al., 2008). In an educational setting, zebrafish are well suited for research (Rana et. al., 2010). Zebrafish develop outside the mother, have synchronous development with a clutch, are transparent, and develop rapidly. These characteristics allow the zebrafish to be easily observed and studied with multiple parallel trials, all in a short amount of time, making the zebrafish a valuable resource for research (Petering, et. al., 2016). How will

the amount of caffeine that the embryos are exposed to affect the development of the zebrafish? Based on previous research it is hypothesized that zebrafish embryos exposed to caffeine will develop numerous deformities and increased mortality, regardless of the concentration of caffeine solution the embryos are exposed to. However, it is also hypothesized that the zebrafish exposed to the highest concentration of caffeine will develop the most abnormalities and have the highest mortality rates.

Materials and Methods

- 1.0 mg/mL, 0.25 mg/mL, 0.05 mg/mL caffeine solutions
- 28.5℃ incubator
- 3x4 well plate and cover
- Disposable 1 mL Pipettes
- Disposable pipette, minimum bore, 1.5 mm for transferring eggs to observation container and manipulating them in the container
- Tape and sharpie marker
- Waste beaker for dead embryos and liquid disposal
- Zebrafish embryos
- Instant Ocean (Control solution)
- Methylene blue (Antiseptic solution)
- Microscope
- Depression slide and cover slip

Procedure

Day 1

1. Obtain embryos from teacher
2. Label well plate with name and class hour. Label rows as control, 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL. Use tape and sharpie marker.
3. Fill each well in the control row with 1 mL of Instant Ocean solution and five drops of methylene blue using a disposable pipette. Fill the other three rows in the same manner with their corresponding caffeine solutions. Be sure to use a different pipette for each solution to avoid mixing solutions. Divide the embryos so there are approximately 10 in each well.
4. Record the numbers of live embryos. Dead embryos should be discarded.
5. Observe the embryos under the dissecting microscope. Record observations.
6. Place well plate with cover on in the 28.5℃ incubator overnight.

Day 2

1. Remove well plate from incubator.
2. Remove dead embryos from all wells in the well plate using disposable pipette. Count and record in data table. Put into waste beaker. **Be careful to only remove the dead embryos.**
3. Count remaining live embryos and record in data table.
4. Remove 0.05 mg/mL caffeine solution from each well in that row. Note: Tilt the plate so the embryos settle and remove liquid from the top, not discarding any living embryos.

5. Replace the 0.05 mg/mL caffeine solution with appropriate fresh stock using a **clean** pipette.
6. Place plate under dissecting microscope and record observations on data sheet. Note/describe any abnormalities.
7. Remove 1-2 embryos and place on the depression slide with cover slip. Observe the embryos under the compound microscope and record observations on data sheet. Repeat steps 4-7 for 0.25 mg/mL and 1.0 mg/mL solutions.
8. Return embryos to well in the plate.
9. Replace Instant Ocean solution in control wells and add five drops of methylene blue to each control well.
10. Place well plate with cover on in the 28.5℃ incubator.

Day 3

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

Day 4.

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

Day 5.

1. Remove well plate from incubator.
2. Carefully observe each well under the dissecting microscope. Record number of live and dead zebrafish as well as observations.
3. Place all zebrafish and embryos into waste container.
4. Clean well plate and remove all labels.

This procedure is from SEPA- UW-Milwaukee.

Data Table 1: Embryos Alive and Dead Exposed to Instant Ocean Solution

Treatment	Well #	# of Embryos	24 hpf		48 hpf		72 hpf		96 hpf	
			# alive	# dead	# alive	# dead	# alive	# dead	# alive	# dead
Control-Instant Ocean solution	1	10	8	2	7	3	6	4	6	4
	2	10	8	2	8	2	6	4	6	4
	3	10	9	1	9	1	8	2	8	2

The data table shows the number of the zebrafish embryos alive and dead living in instant ocean solution at 24, 48, 72, and 96 hpf.

Data Table 2: Embryos Alive and Dead Exposed to 0.05 mg/mL Caffeine Solution

Treatment	Well #	# of Embryos	24 hpf		48 hpf		72 hpf		96 hpf	
			# alive	# dead	# alive	# dead	# alive	# dead	# alive	# dead
0.05 mg/mL Caffeine solution	4	10	8	2	8	2	8	2	8	2
	5	10	9	1	9	1	8	2	8	2
	6	10	9	1	9	1	9	1	9	1

The data table shows the number of the zebrafish embryos alive and dead living in 0.05 mg/mL caffeine solution at 24, 48, 72, and 96 hpf.

Data Table 3: Embryos Alive and Dead Exposed to 0.25 mg/mL Caffeine Solution

Treatment	Well #	# of Embryos	24 hpf		48 hpf		72 hpf		96 hpf	
			# alive	# dead	# alive	# dead	# alive	# dead	# alive	# dead
0.25 mg/mL Caffeine solution	7	10	9	1	9	1	9	1	9	1
	8	10	10	0	10	0	10	0	9	1
	9	10	10	0	9	1	9	1	8	2

This data table shows the number of the zebrafish embryos alive and dead living in 0.25 mg/mL caffeine solution at 24, 48, 72, and 96 hpf.

Data Table 4: Embryos Alive and Dead Exposed to 1.0 mg/mL Caffeine Solution

Treatment	Well #	# of Embryos	24 hpf		48 hpf		72 hpf		96 hpf	
			# alive	# dead	# alive	# dead	# alive	# dead	# alive	# dead
1.0 mg/mL Caffeine solution	10	10	6	4	1	9	0	10	0	10
	11	10	5	5	5	5	5	5	1	9
	12	10	6	4	5	5	3	7	0	10

This data table shows the number of the zebrafish embryos alive and dead living in 1.0 mg/mL caffeine solution at 24, 48, 72, and 96 hpf.

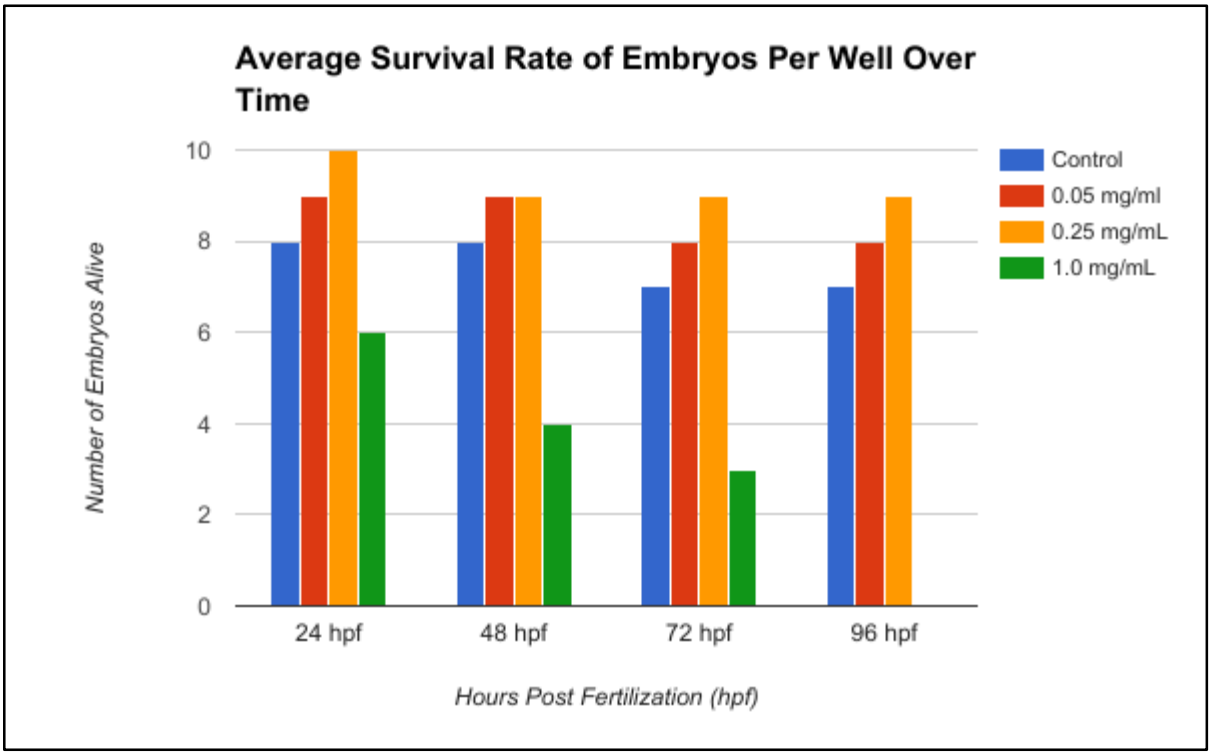
Data Table 5: Unpaired t-test, P- values, and Statistical Significance Between Groups

Sample Group	P-Value	Statistical Significance
72 hpf Control vs. 0.05 mg/ mL	0.089	Not Quite Significant
72 hpf Control vs. 0.25 mg/ mL	0.0058	Very Significant
72 hpf Control vs. 1.0 mg/mL	0.1706	Not Significant
48 hpf Control vs. 0.05 mg/ mL	0.3739	Not Significant
48 hpf Control vs. 0.25 mg/ mL	0.1161	Not Significant
48 hpf Control vs. 1.0 mg/mL	0.0406	Moderately Significant
24 hpf Control vs. 0.05 mg/mL	0.5185	Not Significant
24 hpf Control vs. 0.25 mg.mL	0.0474	Moderately Significant
24 hpf Control vs. 1.0 mg/mL	0.0048	Very Significant

This data table shows the p-values and statistical significance of the results after the unpaired t-

test.

Graph 1: Survival Rates



This graph shows the average number of embryos alive in each well per solution at 24, 48, 72, and 96 hpf.

Figure 1: This shows two embryos 72 hpf in 0.05 mg/mL caffeine solution. One is developed normally, while the other has a bent tail and spine.

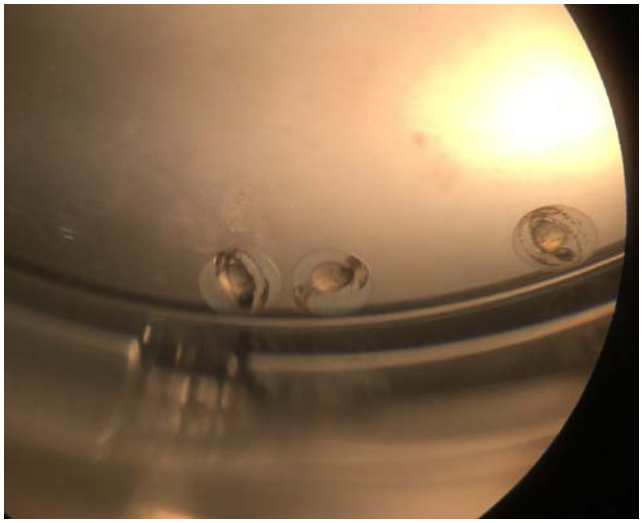


Figure 2: This shows three unhatched embryos 48 hpf control solution. All are developing normally.

Figure 3: This shows two embryos 72 hpf in 0.25 mg/mL caffeine solution. Both have bent tails and enlarged Neither are yolk sacs.

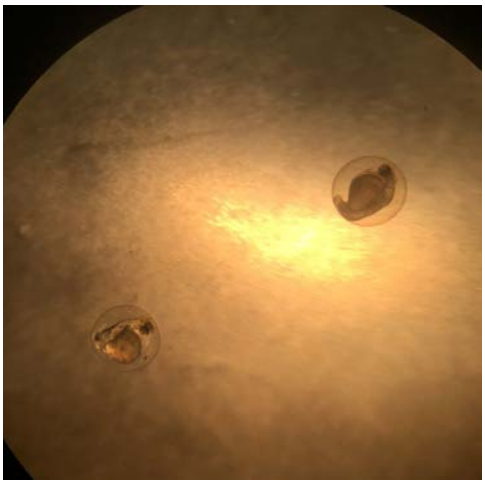


Figure 4: This shows embryos 72 hpf 1.0 mg/mL caffeine solution. hatched, and both have twisted spines and small eyes.

Data / Data Analysis

The statistical test that was used was an unpaired t-test. This was used because two unrelated groups were compared to find the statistical significance between the control and the three other experimental groups. Data table five shows the p-values and statistical significance after the unpaired t-test. There were two moderately significant results and two very significant results, those being 24 hpf control vs. 0.25 mg/mL and 48 hpf control vs. 1.0 mg/mL, and 24 hpf control vs. 1.0 mg/mL and 72 hpf control vs. 0.25 mg/mL, respectively, which means that exposure to high concentrations of caffeine have immediate and can have long term effects on developmental abnormalities and mortality rates. Data table one shows the number of the zebrafish embryos alive and dead living in instant ocean solution at 24, 48, 72, and 96 hpf. Data table two shows the number of the zebrafish embryos alive and dead living in 0.05 mg/mL caffeine solution at 24, 48, 72, and 96 hpf. Data table three shows the number of the zebrafish embryos alive and dead living in 0.25 mg/mL caffeine solution at 24, 48, 72, and 96 hpf. Data table four shows the number of the zebrafish embryos alive and dead living in 1.0 mg/mL caffeine solution at 24, 48, 72, and 96 hpf. Graph one shows the average number of embryos alive in each well per solution at 24, 48, 72, and 96 hpf. Figure one shows two zebrafish embryos 72 hpf living in 0.05 mg/mL caffeine solution. One fish is developed normally, while the other has a bent spine. Figure two shows three unhatched embryos 48 hpf living in instant ocean solution. They are all developing normally. Figure three shows two embryos on 72 hpf living in 0.25 mg/mL caffeine solution. Both embryos have bent tails and spines. Figure four shows two embryos 72 hpf living in 1.0 mg/mL caffeine solution. Neither are hatched, and both embryos have twisted spines and smaller than normal eyes.

Results

In this experiment, zebrafish embryos were exposed to different caffeine solutions, as well as a control instant ocean solution. Development and death rate of the embryos was

observed and compared from 4-5 hpf to 96 hpf. The independent variables in this experiment were the 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL caffeine solutions. The dependent variable was the number of deaths and abnormalities in the zebrafish embryos. The control were the embryos that were only exposed to the instant ocean solution. The constants were the incubator temperature, methylene blue, instant ocean solution, caffeine solutions, amount of solution, and the time of day the solutions were changed and the embryos were observed. All embryos living in instant ocean developed normally. At 24 hpf, the spine and tail were normal, eyes were beginning to form, and there was movement. At 48 hpf, the embryos gained more color in the eyes and body, as well as had more movement and blood flow. One embryo was hatched. At 72 hpf, 11 embryos were hatched. More color was present, and there was more movement. The spines and tails were normal. At 96 hpf, all surviving embryos were hatched. Small fins were visible and there was a great deal of movement. All embryos had normal coloration. The embryos in 0.05 mg/mL caffeine solution developed normally as well, except for one embryo that developed a twisted spine and enlarged yolk sac. The embryos otherwise developed the same as those in instant ocean. The zebrafish in 0.25 mg/mL caffeine solution did not develop normally. At 24 hpf, the embryos had no noticeable abnormalities, but had very little movement. At 48 hpf, the eyes and body had gained color and 15 embryos were hatched. Blood flow was visible, and several had twisted spines. At 72 hpf, all embryos were hatched. Many had twisted spines, and there was little movement. 96 hpf was similar, with little movement and twisted spines. Heartbeats were very slow, and the yolk sacs were enlarged. The zebrafish exposed to 1.0 mg/mL caffeine solution had high mortality and severe abnormalities. At 24 hpf, there was little movement, and the tail and spines were normal. At 48 hpf, the spines were very twisted. Eyes were smaller than normal, and color was lighter than normal. At 72 hpf, the embryos had not changed. None were hatched, and more had died. There was no movement, and blood flow was difficult to observe. At 96 hpf, there was only one living embryo left. The embryo was extremely deformed, with a twisted spine and tail, enlarged yolk sac, and small eyes. The experiment aimed to find the significance that caffeine can have on zebrafish when exposed to caffeine during development. The qualitative and quantitative data showed that caffeine has a significant effect on the survival and development of zebrafish embryos. High concentrations of caffeine can cause extreme deformities and high mortality rates in zebrafish embryos.

Discussion

There was a great deal of data collected in this experiment. The twisted spines, bent tails, enlarged yolk sacs and noticeably slow heart beats were the most noticeable abnormalities seen in the embryos living in 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL caffeine solution. The greater the concentration of caffeine, the more abnormalities were present and the higher the mortality rates were. This data supported the hypothesis that the zebrafish exposed to the highest concentration of caffeine will develop the most abnormalities and have the highest mortality rates. However, the hypothesis that zebrafish embryos exposed to caffeine will develop numerous deformities and increased mortality, regardless of the concentration of caffeine solution the embryos are exposed to was not supported. The embryos exposed to 0.05 mg/mL caffeine and 0.25 mg/mL caffeine had decreased mortality compared to those embryos not exposed to caffeine. With the class time limit, the results could have been affected by human error in recognizing and counting the number of living and dead embryos, as well as

identifying the fertilized embryos on day one. This could have affected the data by giving a false count of alive and dead embryos, as well as counting unfertilized embryos as dead. For future research, it would be important to test more embryos and have more trials to obtain more significant data and more reliable result. The data that was collected leads to the conclusion that it is very important for all people, but especially pregnant mothers to limit caffeine consumption, as it is very likely it could negatively affect the unborn child. This supports most current research, leading to the conclusion that caffeine can have adverse effects on human development and growth.

References

Caffeine Intake During Pregnancy. (2016, September 01). Retrieved January 28, 2017, from http://americanpregnancy.org/pregnancy-health/caffeine-during-pregnancy/

Medicines in my Home: Caffeine and Your Body. (2007). Retrieved January 28, 2017, from http://www.fda.gov/downloads/UCM200805.pdf

Pastore, LM, & Savitz, DA. (1995). Case-control study of caffeinated beverages and preterm delivery. [Abstract]. *American Journal of Epidemiology.* Retrieved January 27, 2017, from https://www.ncbi.nlm.nih.gov/pubmed/7801967.

Petering, David H., Craig Berg, Henry Tomasiewicz, Michael Carvan, Louise Petering, and Renee Hesselbach. (2003)."Zebrafish as Models: Studying the Effects of Environmental Agents on Human Health." 1-96.

Petering, D. H., Berg, C., Tomasiewicz, H., Carvan, M., Hesselbach, R., & Petering, L. (2003). Zebrafish As Models: Studying The Effects Of Environmental Agents On Human Health . Retrieved January 27, 2017, from http://guides.library.uwm.edu/ld.php?content_id=2010971

Pietrangelo, A. (2014, October 22). 17 Effects of Caffeine on the Body. Retrieved January 27, 2017, from http://www.healthline.com/health/caffeine-effects-on-body

Rana, N., Moond, M., Marthi, A., Bapatla, S., Sarvepalli, T., Chatti, K., & Challa, A. K. (2010). Caffeine-Induced Effects on Heart Rate in Zebrafish Embryos and Possible Mechanisms of Action: An Effective System for Experiments in Chemical Biology [Abstract]. ZEBRAFISH, 7(1), 69. doi:10.1089=zeb.2009.0631

Veldman, M. B., & Lin, S. (2008). Zebrafish as a Developmental Model Organism for Pediatric Research [Abstract]. Pediatric Research. doi:10.1203/PDR.0b013e318186e609

Whiteman, H. (2015, October 28). Caffeine: how does it affect our health? Retrieved January 27, 2017, from http://www.medicalnewstoday.com/articles/271707.php