The Effects of Caffeine on the Development of Zebrafish

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Abstract:

Usually during pregnancy, the most important concern for a mother is doing what they can to care for the child they will soon have. Most people will quit the habits that are known to be harmful for their child (teratogens). However, some people find it difficult to give up certain habits like consuming caffeine. Some people still consume it, because they may think that a little bit does not have a negative impact on the fetus. The purpose of studying the effects of caffeine on embryo development is to discover what that minimal amount of caffeine can do, regarding development. The zebrafish were the test subjects due to their similarity to human organ systems. We used 4 different concentrations to see the effects of no caffeine versus higher concentrations of caffeine. In every concentration of caffeine, we observed some negative development signs. In the higher concentrations some of the embryos looked solid in color and did not show organs that are normally displayed in development. In the lower concentrations some of the hatched zebrafish showed spinal deformities. The spine of the fish looked inverted and curved the opposite way a normal zebrafish would.

Introduction:

With the intent to study the effect of caffeine on early development, we used Zebrafish because they are excellent models for representation of effects on human life due to their transparency and similar organ systems. The variable being tested in this experiment is caffeine and how that substance specifically affects embryo development. Caffeine is a central nervous system stimulant, and studies have shown that exposure to it during pregnancy can lead to increased abnormalities, including decreased fertility and low birth weight (Qian, 2018). One study shows that pregnant mice treated with a moderate dosage of caffeine resulted in defective embryo

development and uterine receptivity (Qian, 2018). Caffeine also has a significant impact on brain function. Exposure to caffeine, which is a blocker of adenosine receptors, has been suggested as a developmental risk factor (Menezes, 2018). Disruption of adenosinergic signaling during early stages of life can change the stable neural network formation and ultimately, can lead to an increase in the likelihood of seizures (Menezes, 2018). In an experiment on zebrafish, morpholines (adenosine receptor antagonist) were utilized during the embryonic phase of the fish. The block of these receptors increased the mortality rate and caused an elevated rate of malformations (Menezes, 2018). Lastly, depending on the amount of caffeine tested, caffeine greatly increased zebra fish's heartbeats per minute (Abdelkader, 2013). However, caffeine supposedly does not affect survival, but it can delay hatching and promote oxidative stress (Abdelkader, 2013). The hypothesis for the experiment being conducted is that caffeine will negatively impact the zebrafish embryos. We expect that as the concentration of caffeine is increased, the amount of hatching will decrease proportionately and many negative effects will arise in the embryos.

Materials and Methods:

Materials:

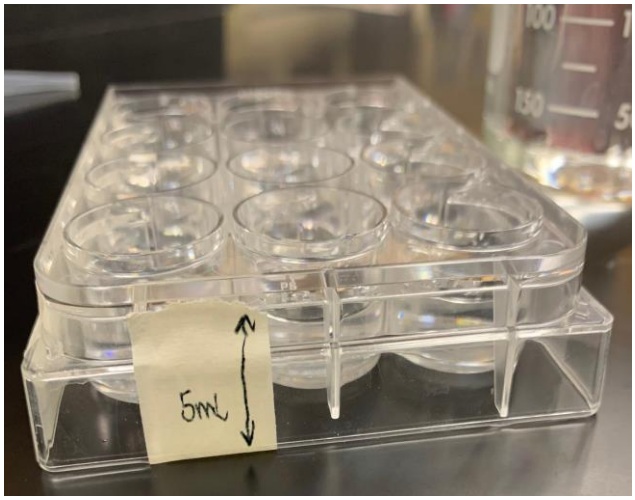
- 3 beakers of stock solutions of caffeine (0.05 mg/mL, 0.25 g/mL, 1.0 g/mL)
- 1 beaker for dead embryos and excess liquid disposal
- 1 bottle of embryo media solution (control)
    - Instant Ocean was mixed to a concentration of 200 mg/L to make embryo media solution
- 4 disposable pipettes (for each concentration of caffeine, 1 mL)
- 1 multi-well plate, or Falcon dish
- 1 incubator set at 28.5 degrees Celsius
- 1 compound microscope

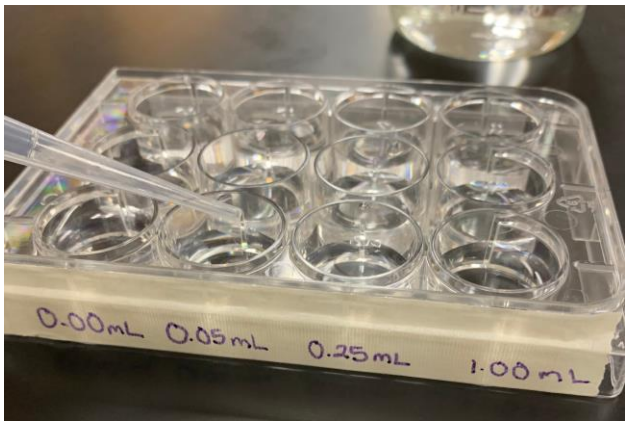
- 1 permanent marker (Sharpie)
- 60 zebrafish embryos

Procedure:

Day 1:

1. Obtain zebrafish embryos and gather all materials.
2. Make the caffeine solutions and the control in separate beakers and label them with a permanent marker 0.0 mg/ mL (control), 0.05 mg/mL, 0.25 mg/mL, and 1 mg/mL based on their caffeine concentration.
    a. 0.0 mg/mL: add 0 mL of stock and 50 mL of embryo media solution
    b. 0.05 mg/mL: add 2.5 mL of stock and 47.5 mL of embryo media solution
    c. 0.25 mg/mL: add 12.5 mL of stock and 37.5 mL of embryo media solution
    d. 1.0 mg/mL: add 50 mL of stock and 0 mL of embryo media solution
3. Label each row of the Falcon dish with the specific concentration tested.





4. Place 3mL of each solution into the wells in their designated row.
5. Use a pipette to place 5 zebrafish in each well and cover the Falcon dish with its lid. There should be 15 zebrafish per concentration (3 wells for each concentration and 5 zebrafish per well)
6. Place the Falcon dish into the incubator and leave it there overnight.

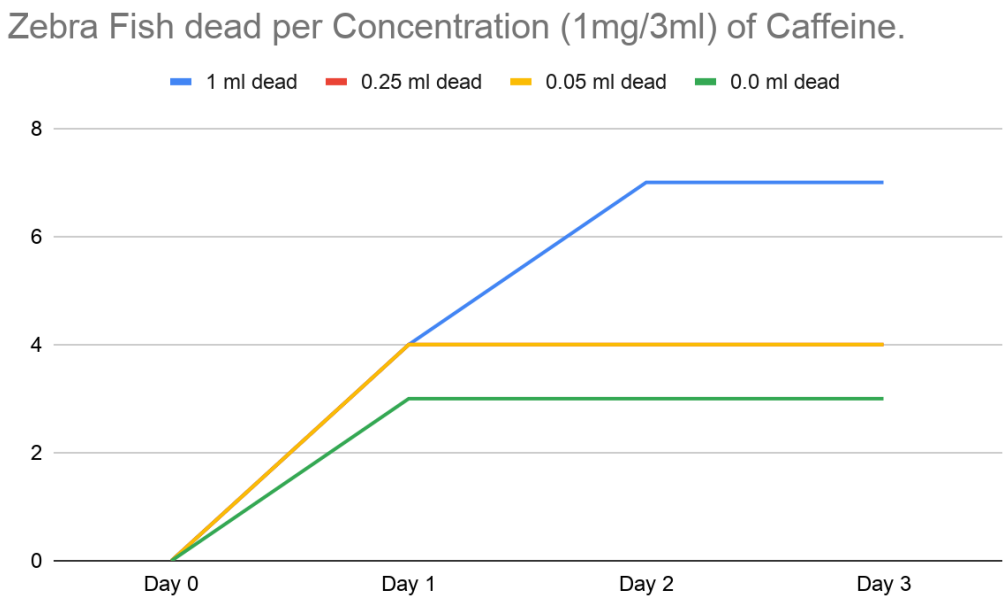
Days 2-4:

1. Use a microscope to record the number of zebrafish living and dead.
2. Make note of any other qualitative observations. Take pictures of the zebrafish and label which development stage they are in.
3. Remove all liquid and dead embryos from all of the wells. Leave living zebrafish inside the well.
4. Add 3 mL of the differing caffeine solutions into their set well row.
5. Place Falcon Dish once again into the incubator. Leave it there.

6. After the experiment was done, we used a Fisher Exact test to assess statistical significance between the caffeine treatments.

Results:

The hypothesis stated that as the concentration of caffeine increases, the rate of hatching will decrease based on those concentrations. The independent variable in this experiment is the concentration of each caffeine solution, since that is the factor in the experiment that is manipulated. The dependent variable was the amount of zebrafish embryos that hatched and had deformities, because it depends on the concentration of caffeine in their environment. The control was the ocean/embryo media solution, meaning it has no caffeine. As seen in Figure 1, the control group and both experimental groups 0.05 mg/mL and 0.25 mg/mL all ended with 11 zebrafish alive and 4 dead. The 1.0mg/mL experimental group had fewer zebrafish alive and more dead. As for hatching, more than half of the embryos hatched in the control solution (6 out of 11). Lastly, in the 0.05 mg/mL solution, all 11 of the embryos hatched. In the higher concentrations, the amount of hatched embryos decreased greatly. In the 0.25 mg/mL group, 3 embryos hatched and one was in the process of doing so out of 11 embryos, meaning around ⅓ hatched. Lastly, the 1.0 mg/ml concentration, none of the living embryos hatched. This shows there was a slight peak from the control to the 0.05 mg/mL concentration but after, it was a sharp decline.



Several deformities were observed in the zebrafish such as tails wrapped up with their bodies (bent), yolk sac edemas, and embryos out of their membrane, but not hatched. All of these deformities were present in the 1.0 mg/mL caffeine solution, which appeared to have especially weak and fragile membranes. Additionally, it appeared that the embryos in the lower concentrations moved more, whereas those in higher concentrations seemed more constricted and moved less. Those present in higher concentrations also had stranger shapes.

Number of Total Zebrafish Embryos Alive (A) vs. Dead (D) From the Beginning to the End of the Experiment:

Day:	Hours Post Fertilization (hpf)	Control Group 0.0 mg/mL	Experimental Group 0.05 mg/mL	Experimental Group 0.25 mg/mL	Experimental Group 1.0 mg/mL
Day 0	0 hpf	15 A/ 0D	15 A/0D	15A/0D	15A/0D
Day 1	24 hpf	12 A/ 3D	11 A/ 4D	11A/ 4 D	11A/ 4D

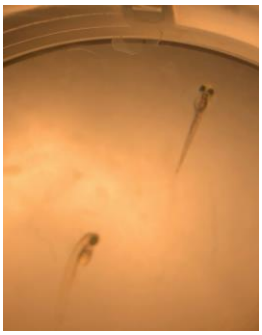
Day 2	48 hpf	11 A/ 4 D	11A/ 4D	11A/4 D	8 A/7D
Day 3	72 hpf	11 A/4 D	11A/4D	11 A/4D	8A/ 7D

Figure 1

Number of Zebrafish Embryos Hatched (H) vs. Unhatched (U) From the Beginning to the End of the Experiment:

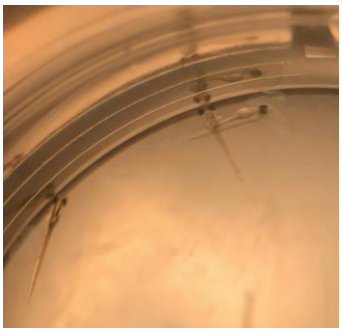
Day:	Hours Post Fertilization (hpf)	Control Group 0.0 mg/mL H/U	Experimental Group 0.05 mg/mL H/U	Experimental Group 0.25 mg/mL H/U	Experimental Group 1.0 mg/mL H/U
Day 1	0 hpf	12 U	11 U	11 U	11 U
Day 2	24 hpf	11 U	11 U	11 U	8 U
Day 3	48 hpf	6 H/ 5 U	11 H	3 H, 1 in the process of hatching/ 7 U	8 U

Figure 2



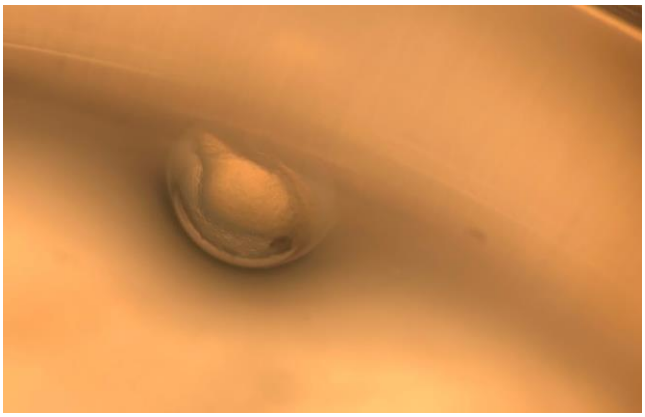
This is the 0.0 concentration on the 3rd day, that shows normal, healthy zebrafish after they hatched.

This is the 0.05 ml concentration on the 3rd day, that shows normal, healthy zebrafish after they hatched.



This is the 0.25 ml concentration on the 3rd day, 3 embryos hatched and one is unhatched, but shows deformities.

This is the 1.0 ml concentration on the 3rd day, these embryos didn't hatch and were more condensed than the other concentrations before hatching.



This is what happened to most of the high concentration embryos (1.0 and 0.25 concentrations) before they died. They turned a solid like texture and none of the usually visible organs were shown.

Data Analysis:

Fisher's exact test is a statistical test used to determine if there are nonrandom associations between two variables. This test works exceptionally well with this experiment since the main goals are to compare if the hatching rates and number of deformities of the different groups differ significantly. When the test was used to compare the amount of hatching for the control versus the 0.05 mg/mL concentration, the p value ended up being 0.0351. Because p is less than 0.05, this result is significant. This shows that there is a very big impact between no caffeine and a minimized amount of it, as the hatching amount significantly increases from the control (went from more barely half of the embryos hatched to all of them hatched). We also calculated the significance of the amount of hatching using the control and the 1.0 mg/mL concentration; the result also turned out significant with its p-value being 0.0181, less than 0.05. This shows the control that contains no caffeine and the highest concentrated caffeine concentration had significantly different impacts on the hatching rate. In the control group, barely over half of the embryos hatched, whereas none hatched in the 1.0 mg/mL solution; this is a significant decrease in hatching amount. However, the statistical value between the control and the 0.25 mg/mL concentration for hatching was 0.387, showing the relationship is not significant. Compared to the 0.25 mg/mL solution, the control only had 3 more completely hatched embryos and 2 less unhatched, which is not a significant decrease.

Discussion:

Overall, this experiment showed us there were more deformities, less hatching, and more fatalities as the concentration of caffeine increased in a zebrafish's environment. These were the general trends shown within our data. Our original hypothesis was that the amount of hatching would decline as the concentration of caffeine increases. It seemed that with a small concentration of caffeine, the hatching process became accelerated. However, once the concentration was larger, it was detrimental to the hatching process and slowed it down, as our research and hypothesis said. There was a nonsignificant result between the control and the 0.25 mg/ mL concentration; this could be because after the peak of hatching with the 0.05 mg/ml solution, the higher concentration begins to inhibit the hatching process and bring the hatching amount down to what it was previously before.

Another part of our hypothesis was that the embryos would experience negative effects due to the caffeine. With higher concentrations of caffeine the embryos started to look firmer in texture, rather than a transparent membrane that visually shows all of the embryo's organs. A large majority of the embryos subjected to caffeine during development experienced some deformities; however, most were observed higher concentrations, especially the 1.0 mg/mL solution. In the 1.0 mg/mL concentration, all of the embryos experienced deformities. This also supports our hypothesis, because the deformities seemed to arise in the greater concentrations like we predicted.

One limitation in this experiment could be not having control over extraneous variables that might bias our results. Some of the embryos may not have already been in the best conditions; other tasks (such as putting them into the incubator,), could not have been the best

regulated temperature for some of the embryos, which could have caused death. An error encountered in this experiment did impact the data. For well number three on day two for the 1.0 mg/mL solution, there were 3 zebrafish embryos alive. However, during the extraction process of the dead embryos, three alive embryos burst. Their membranes and outer coverings were extremely fragile. This instance reduced the number of embryos in that well from three to zero, which could have had an impact on our data statistics.

All mothers who care about the health of their baby should be careful with what they consume during pregnancy. With the consumption of caffeine in great amounts, the embryo may have negative health effects including organ damage and deformities. However, because zebrafish and humans aren't entirely identical, more studies and more research would greatly contribute more to the idea of how drastic and extensive the effects of caffeine consumption are on embryos and developing organisms.

Resources

Qian, Jingjing, et al. “Caffeine Consumption during Early Pregnancy Impairs Oviductal Embryo Transport, Embryonic Development and Uterine Receptivity in Mice.” *Biology of Reproduction*, Oxford University Press, 1 Dec. 2018, www.ncbi.nlm.nih.gov/pubmed/29982366.

Menezes, Fabiano Peres, et al. “Transient Disruption of Adenosine Signaling During Embryogenesis Triggers a Pro-Epileptic Phenotype in Adult Zebrafish.” *Molecular Neurobiology*, U.S. National Library of Medicine, Aug. 2018, www.ncbi.nlm.nih.gov/pubmed/29327202.

Abdelkader, Tamer Said, et al. “Exposure Time to Caffeine Affects Heartbeat and Cell Damage-Related Gene Expression of Zebrafish Danio Rerio Embryos at Early Developmental Stages.” *Journal of Applied Toxicology : JAT*, U.S. National Library of Medicine, Nov. 2013, www.ncbi.nlm.nih.gov/pubmed/22886764.