Effects of Caffeine on the Embryonic Development of Zebrafish (Danio rerio)

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Abstract

Caffeine is a toxin that most people consume everyday without thinking twice, despite the possible hazards it can cause. An intake of caffeine can be especially dangerous for pregnant woman because it can inhibit proper development of the child they are carrying. In search of which stage of development is the most critical and that caffeine should be avoided most, an experiment was conducted using Danio rerio, or zebrafish. Zebrafish are appropriate models because they are vertebrates like humans. Zebrafish's clear embryos and fast rate of development allow research to be done effectively. This study exposed 129 zebrafish embryos to 1.00 mg/mL of caffeine solution at different periods post fertilization to test which period was most critical to development. Those zebrafish that were exposed to caffeine were then compared to 30 zebrafish in the control group that were never exposed to caffeine. Out of the 129 zebrafish 32 were exposed to 1.00 mg/mL of caffeine only on day one, 37 were exposed only on day two, 30 were exposed only on day three, and 30 were exposed on all three days. All of the embryos that were exposed to caffeine at some point during the experiment showed signs of severe deformities. The results of this experiment showed no signs that one period of development was more crucial than another because all embryos exposed to caffeine had abnormalities despite the timing of exposure. This experiment is significant to human health and shows that people should monitor their caffeine intake.

Introduction

Caffeine used to just be found in a simple cup of tea or coffee, but it has become a fad that can be found in almost any form. Caffeine is now popularly found in energy drinks (such as Red Bull and Monster), Jolt gum, "Stay Awake" pills, and the strongest form of all pure powder. These new products as convenient as they may be are causing thousands of overdoses, addiction and withdrawal becoming a serious public health problem. There has even been deaths reported due to the dangerous effects of caffeine. Many people are very dependent on this drug to give them that extra boost of energy to get through the day. So many people rely on it that it is the most popular psychoactive agent consumed worldwide. It is estimated that 85 percent of people in the United States consume caffeine at least once a day (Cha, 2015). Some of these consumers are pregnant women that are not only inflicting possible hazards on themselves, but also the innocent child in their womb. Caffeine is able to effect the child in the womb by traveling across the placenta. Caffeine is much more dangerous for a newly developing child than the mother because the child is not capable of controlling the amounts of caffeine in its body since the baby's metabolism cannot fully metabolize the caffeine. There have been numerous animal studies that have exhibited caffeine causing reproductive problems such as reduced fertility, premature labor, birth defects, preterm delivery, and increase the risk of low birth weight offspring (Caffeine Intake During Pregnancy, 2015). In moderation consuming caffeine as an adult and not pregnant does not appear so harmful, but in the last couple of years hospitals and

poison centers across the country have reported an increase in emergencies caused by caffeine overdoses (Cha, 2015).

With this take over of caffeine in our society today, it is important to know exactly what the effects this drug can have on people. Many forms of research have been developed to uncover this mystery, such as experimentation on zebrafish. Using zebrafish embryos as a model for research has been growing in popularity because zebrafish embryos are vertebrates they can be used to understand all vertebrates including people making them a powerful tool. Zebrafish embryos are a more convenient means of testing because they develop from the moment that sperm fertilizes an egg and allow researchers to observe the zebrafish embryo transform into a fish under a microscope because the eggs are clear and develop outside of the mother's body (Badman, Dougan, Greenberg, Heindel, Mathews, Trevarrow). Zebrafish allow scientists to study the development effects caffeine has which makes them such a powerful tool since it is difficult to monitor the progress in humans because their offspring are carried inside the body. By studying zebrafish in the lab, we are able to eliminate many possible variables that could be present in the internal environment of a mother. Zebrafish have the ability to develop at a very high rate, growing from just a single cell to an organism that is recognizable as a microscopic fish within 24 hours. This fast rate of development allows the researcher to see the effects much faster than mice which take 21 days (Badman et al.). When zebrafish eggs are exposed to the toxin, researchers are able to closely monitor development and observe abnormalities under a microscope. These exposed fish are then compared to a controlled set of fish. Another reason why the use of zebrafish is ideal for research on the effects of various toxins on development is there is an abundance of knowledge on what is normal for zebrafish. This allows scientists to make a clear comparison and come to a more confident conclusion of the adverse effects of chemical exposure (Hill, Teraoka, Heideman, Peterson, 2005).

It has been calculated the average cup of brewed coffee contains 163 mg of caffeine (Brewed Coffee). Knowing that a large number of people consume more than just one cup of brewed coffee in a day, it was predetermined that the concentration of caffeine used in experimentation should be raised greatly to 1.00 mg/mL to match the effect of multiple cups being consumed in one day. Previous experiments show that this high concentration of caffeine has proven to be challenging to the healthy development of zebrafish, but many are still able to survive despite the abnormalities it causes (Brunning, 2014). It is also known that certain periods of development are more crucial for different parts of the body to develop. This leads to the question of which period of development has the greatest impact on development? It is hypothesized that the exposure to caffeine in the beginning stages of development, day one, will have the largest impact on the development of the zebrafish embryos. To test this hypothesis an experiment exposing separate wells of zebrafish to the same concentration of caffeine on different days will be conducted. The death rate and abnormalities from each variation of exposure will be compared to show which day is most crucial.

Materials and methods

Materials:

- 1 bottle of 1.00mg/mL caffeine solution (Variable solution)
- 1 bottle Instant Ocean/Embryo Media Solution (Control solution)
- Methylene Blue (Antiseptic solution)
- 5 disposable pipettes (1.5mm for transferring and manipulating eggs)
- 2 disposable 1 mL pipettes (for each day testing)
- 1 waste beaker for dead embryos and liquid disposal
- Labeling tape
- Labeling marker (Sharpie)
- 2 3x4 falcon plates with 12 wells
- Microscope depression slide with coverslip
- Dissecting and compound microscopes
- 28.5℃ Incubator

Safety Precaution:

- Wear gloves when handling the concentrated caffeine solution.

Procedure:

Day 1

1. Obtain two well plates and label each column (composed of three wells) with the day that they will be exposed to the caffeine solution using the Sharpie and tape.
2. Add 1 mL of the control solution (instant ocean) into every well except for the three wells in column 2 (the only day one column) and the three wells in column 4 (the all three days column).
3. Add 2 mL of the 1.00 mg/mL caffeine solution into all three wells of column 2 and all three wells in column 4.
4. Place 10 Zebrafish embryos into each well.
5. Add 1 drop of methylene blue to all wells to prevent bacteria growth.
6. Carefully observe each well under the dissecting microscope and record exact numbers and observations of live embryos on the data sheet and discard dead embryos.
7. Place both falcon plates in the 28.5°C incubator overnight.

Day 2

1. Remove both falcon plates from incubator.
2. Carefully observe each well under the dissecting microscope and record the number dead and alive on the data sheet.
3. Remove all dead embryos from both plates using a fine-tipped disposable pipette and dispose them into the waste beaker.

4. Remove the solutions from all of the wells by tilting the plate so the embryos settle and remove the liquid from the top with a disposable pipette being careful to not discard any living embryos.
5. Add 1 mL of the control solution (instant ocean) to each well except for the three wells in column 3 (the only day two column) and the three wells in column 4 (the all three days column).
6. Add 1 mL of the 1.00 mg/mL caffeine solution into the wells in columns 3 and 4.
7. Add 1 drop of methylene blue to each well.
8. Place plate under dissecting microscope and record qualitative observations of development and abnormalities on data sheet.
9. Remove 1 to 2 embryos and place them on the depression slide with coverslip. Observe the embryos using the compound microscope. Record observations on data sheet. This is also a good time to take pictures of the embryos.
10. Return the embryos to their well in the plate.
11. Place both falcon plates in the 28.5°C incubator overnight.

Day 3

1. Remove both falcon plates from the incubator.
2. Carefully observe each well under the dissecting microscope and record the number dead and alive on the data sheet.
3. Remove all dead embryos from both plates using a fine-tipped disposable pipette and dispose them into the waste beaker.
4. Remove the solutions from all of the wells by tilting the plate so the embryos settle and remove the liquid from the top with a disposable pipette being careful to not discard any living embryos.
5. Add 1 mL of the control solution (instant ocean) to each well except for the three wells in column 1 on the second well plate (the only day three column) and the three wells in column 4 on the first well plate (the all three days column).
6. Add 1 mL of the 1.00 mg/mL caffeine solution into the wells in column 4 on the first well plate and column 1 on the second well plate.
7. Add 1 drop of methylene blue to each well.
8. Place plate under dissecting microscope and record qualitative observations of development and abnormalities on data sheet.
9. Remove 1 to 2 embryos and place them on the depression slide with coverslip. Observe the embryos using the compound microscope. Record observations on data sheet. This is also a good time to take pictures of the embryos.
10. Return the embryos to their well in the plate.
11. Place both falcon plates in the 28.5°C incubator overnight.

Day 4

1. Remove both falcon plates from the incubator.
2. Carefully observe each well under the dissecting microscope and record the number dead and alive along with observations of development and abnormalities on the data sheet.
3. Place all living Zebrafish from the control column into container specified by teacher.
4. Clean the well plate and remove all labels.

Procedure is from SEPA- UW-Milwaukee.

Data

Data Table 1: Control Zebrafish Number of Dead and Alive Zebrafish in a 72 hpf Time Period

Treatment	Well Number	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization	
			Number Alive	Number Dead	Number Alive	Number Dead	Number Alive	Number Dead
Control	1	10	5	5	5	0	5	0
	2	10	7	3	7	0	7	0
	3	10	6	4	6	0	6	0

Data table 1 shows how many zebrafish embryos were alive after a 24 hpf period, 48 hpf period, and a 72 hpf period. The zebrafish in wells 1, 2, and 3 were the control group in the instant ocean environment to be used to make comparisons between the variable groups. These fish were never exposed to caffeine and had a high survival rate with more than half of the zebrafish surviving in all three wells.

Data Table 2: Number of Dead and Alive Zebrafish Exposed to 1 mg/mL Caffeine Only on Day One in a 72 hpf Time Period

Day of Exposure to 1.00 mg/mL of Caffeine Solution	Well Number	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization	
			Number Alive	Number Dead	Number Alive	Number Dead	Number Alive	Number Dead
Only Day 1	4	11	9	2	9	0	9	0
	5	10	4	6	4	0	4	0
	6	11	9	2	9	0	9	0

Data table 2 shows how many zebrafish embryos were alive after a 24 hpf period, 48 hpf period, and a 72 hpf period. The zebrafish in the wells 4, 5, and 6 were exposed to 1.00 mg/mL of caffeine only on day one. On day two and three the fish in these three wells were in the instant ocean environment. The zebrafish exposed to caffeine only on day one had a high survival rate.

Data Table 3: Number of Dead and Alive Zebrafish Exposed to 1 mg/mL Caffeine Only on Day Two in a 72 hpf Time Period

Day of Exposure to 1.00 mg/mL of Caffeine Solution	Well Number	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization	
			Number Alive	Number Dead	Number Alive	Number Dead	Number Alive	Number Dead
Only Day 2	7	16	12	4	12	0	12	0
	8	11	9	2	9	0	9	0
	9	10	6	4	6	0	5	1

Data table 3 shows how many zebrafish embryos were alive after a 24 hpf period, a 48 hpf period, and a 72 hpf period. The zebrafish in wells 7, 8, and 9 were exposed to 1.00 mg/mL of caffeine solution only on day two. On day one and day three these three wells were in the instant ocean environment. The zebrafish exposed on only day two show a high survival rate.

Data Table 4: Number of Dead and Alive Zebrafish Exposed to 1 mg/mL Caffeine Only on Day Three in a 72 hpf Time Period

Day of Exposure to 1.00 mg/mL of Caffeine Solution	Well Number	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization	
			Number Alive	Number Dead	Number Alive	Number Dead	Number Alive	Number Dead
Only Day 3	1	10	9	1	9	0	9	0
	2	10	6	4	6	0	1	5
	3	10	10	0	9	1	0	9

Data table 4 shows how many zebrafish embryos were alive after a 24 hpf period, a 48 hpf period, and a 72 hpf period. The zebrafish in wells 1, 2, and 3 in the second plate were exposed to 1.00 mg/mL of caffeine solution only on day three. On day one and two these three wells were in the instant ocean environment. The zebrafish exposed on only day three show a high survival rate only in one well and a very low survival rate in two of the wells.

Data Table 5: Number of Dead and Alive Zebrafish Exposed to 1 mg/mL Caffeine All Three Days in a 72 hpf Time Period

Day of Exposure to 1.00 mg/mL of Caffeine Solution	Well Number	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization	
			Number Alive	Number Dead	Number Alive	Number Dead	Number Alive	Number Dead
All 3 Days	10	10	4	6	4	0	3	1
	11	10	2	8	2	0	2	0..
	12	10	3	7	3	0	2	1

Data table 5 shows how many zebrafish embryos were alive after a 24 hpf period, a 48 hpf period, and a 72 hpf period. The zebrafish in wells 10, 11, and 12 were exposed to 1.00 mg/mL of caffeine solution on all three days. The zebrafish exposed on all three days show a very low survival rate with less than half of the zebrafish embryos surviving.

Figure 1: Bar Graph of the Survival Rates

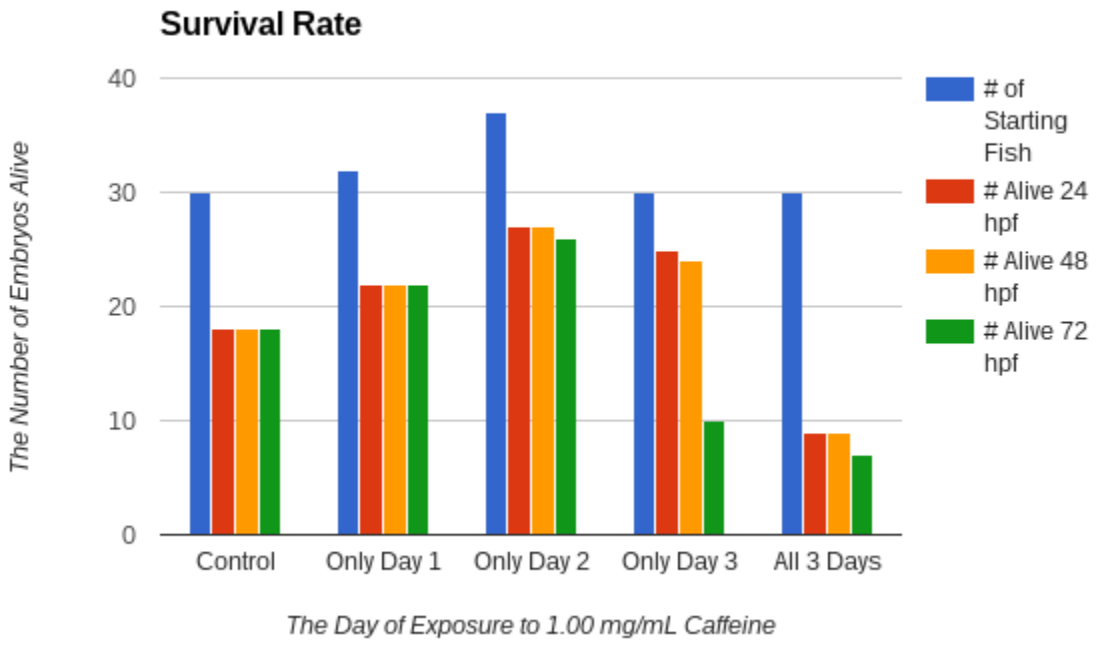


Figure 1 is a bar graph that shows visually how the survival rates of the different periods of exposure vary.

Figure 2 Figure 3 Figure 4

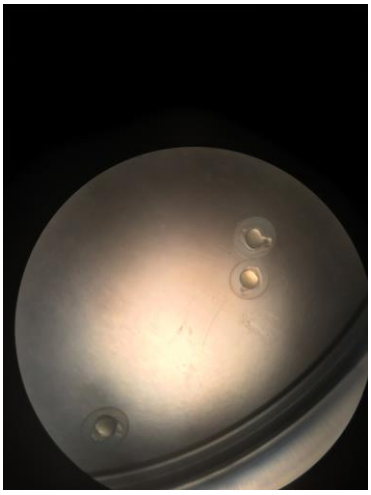


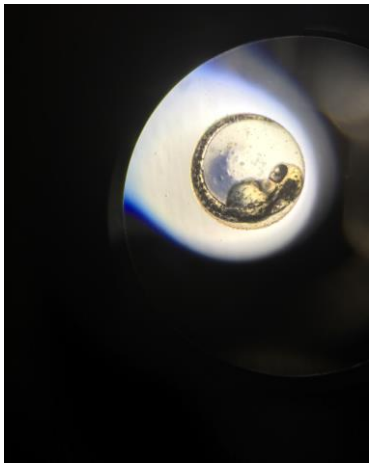




Figure two is an image of three zebrafish embryos at 24 hpf that were exposed to caffeine only on day one. Figure three is an image of a hatched embryo 72 hpf that was exposed to caffeine only on day 3. The zebrafish in this image has an enlarged stomach and rounded tail. Figure four is an image of a zebrafish 72 hpf that was exposed to caffeine on all three days. This zebrafish shows clear signs of the lack of cell differentiation that was noticed in the exposed fish.

Figure 5 Figure 6 Figure 7





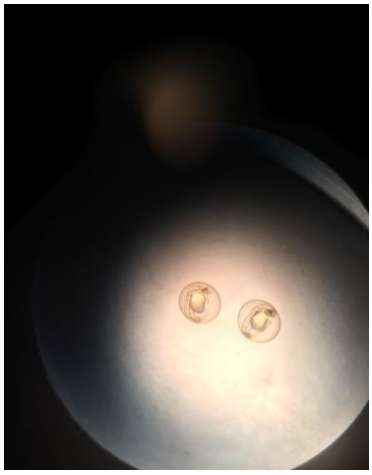


Figure 5 is an image of a zebrafish 72 hpf exposed to caffeine only on day two. This zebrafish has the abnormal bend in the tip of the tail. Figure six is an image of a zebrafish with normal development from the control group 48 hpf. Figure seven is an image of two embryos 48 hpf that were exposed to caffeine only on day two.

Figure 8

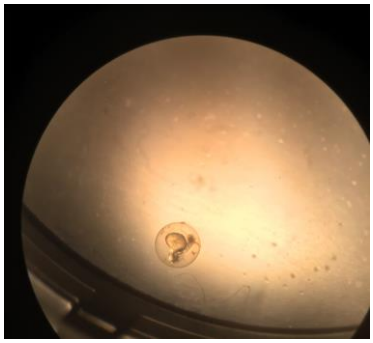


Figure eight is an image of a zebrafish exposed to caffeine all three days 72 hpf. The image was viewed from under a dissecting microscope which is why it is not as clear, but despite the low quality image an abnormally large stomach is still visible.

Data Analysis

T-tests were used to analyze the data found. A T-test is a statistical comparison of two groups. A T-test is used to examine whether the two groups are indeed different by evaluating the differences in data and calculating the p-value. The p-value, or alpha level, is the probability the difference found in the data was not just due to chance, but that the difference is in fact significant. A p-value of less than 0.05 is the goal level for this type of research and means that the probability of the difference seen in the data is due to random error is less than 5%.

Data Table 6: The p-Value and Statistical Significance Between Experiments Found Using the T-test During 24 hpf Period

Comparison	p-Value	Significance
Control vs. Only Day 1	0.4918	Not Statistically Significant
Control vs. Only Day 2	0.1757	Not Statistically Significant
Control vs. Only Day 3	0.1550	Not Statistically Significant
Control vs. All 3 Days	0.0213	Statistically Significant

Data table six shows the p-values found using the unpaired T-test comparing the control group to all four experimental groups after 24 hpf. The comparisons were made between the number of alive zebrafish from the control group and the number alive from the experimental groups exposed to 1.00 mg/mL caffeine on different days. These comparisons were not statistically significant except for the comparison between the control group and the group exposed to caffeine all three days.

Data Table 7: The p-Value and Statistical Significance Between Experiments Found Using the T-test During 48 hpf Period

Comparison	p-Value	Significance
Control vs. Only Day 1	0.4918	Not Statistically Significant
Control vs. Only Day 2	0.1757	Not Statistically Significant
Control vs. Only Day 3	0.1583	Not Statistically Significant
Control vs. All 3 Days	0.0213	Statistically Significant

Data table seven shows the p-values found using the unpaired T-test comparing the control group to all four experimental groups after a 48 hpf. The comparisons were made between the number of alive zebrafish from the control group and the number alive from the experimental groups exposed to 1.00 mg/mL caffeine on different days. These comparisons were not statistically significant except for the comparison between the control group and the group exposed to caffeine all three days.

Data Table 8: The p-Value and Statistical Significance Between Experiments Found Using the T-test During 72 hpf Period

Comparison	p-Value	Significance
Control vs. Only Day 1	0.4918	Not Statistically Significant
Control vs. Only Day 2	0.2746	Not Statistically Significant
Control vs. Only Day 3	0.4107	Not Statistically Significant
Control vs. All 3 Days	0.0053	Very Statistically Significant

Data table eight shows the p-values found using the unpaired T-test comparing the control group to all four experimental groups after a 72 hpf. The comparisons were made between the number of alive zebrafish from the control group and the number alive from the experimental groups exposed to 1.00 mg/mL caffeine on different days. These comparisons once again were not statistically significant except for the comparison between the control group and the group exposed to caffeine all three days. This suggests that exposing zebrafish to caffeine all three days greatly increases the death rate.

Data Table 9: The p-Value and Statistical Significance Between Dependent Experiments Found Using the T-test

Comparison	p-Value	Significance
Only Day 1 vs. Only Day 2	0.7886	Not Statistically Significant
Only Day 1 vs. Only Day 3	0.3373	Not Statistically Significant
Only Day 1 vs. All 3 Days	0.0660	Not Quite Statistically Significant
Only Day 2 vs. Only Day 3	0.2533	Not Statistically Significant
Only Day 2 vs. All 3 Days	0.0081	Very Statistically Significant
Only Day 3 vs. All 3 Days	0.7449	Not Statistically Significant

Data table nine shows the p-Values found using the unpaired T-test comparing the amount of zebrafish alive on the different exposure days to test the significance of exposing zebrafish to caffeine at different stages to find if there is a stage of development when zebrafish embryos are more vulnerable. According to the p-values found none of the death rates varied enough to show that one stage is more vulnerable than the other.

Results

In this experiment the control group was never exposed to caffeine. The control group lived in a variable free environment of the instant ocean solution to be used for comparing the experimental groups. The independent variables in this study were the 1.00 mg/mL concentration of caffeine and the timing of the exposure. The dependent variable was the number of deaths and abnormalities that resulted from the caffeine solution. The constants used in this experiment were the incubator temperature of 28.5℃, the one drop of methylene blue, the amounts of solution, and the time of day fish were exposed to the solutions. These constants are what allows us to rule out possible outside variables and determine whether the caffeine alone has an effect or not.

When the embryos were placed into the wells, they were examined and all were determined to be healthy and fertilized and at about the 5 hpf stage. The fish were then later examined at the 24 hpf period. Out of the 30 embryos in the control group 12 of the embryos were determined to be dead based on the bacteria found inside the chorion, but the rest of the embryos were developing at a healthy rate. In the Only Day 1 group 22 of the 32 embryos had survived. Their survival was indicated by some quick twitching movement in the chorion, but the bacteria growth inside of the other 10 embryos indicated that those other 10 had died. These fish were already showing some abnormalities with overly inflamed stomachs and slightly bent tails. Ten of the 37 embryos exposed to 1.00 mg/mL caffeine solution only on day 2 had died. The fish that were alive closely resembled the fish in the control group and were at a healthy rate of development. The embryos that were alive in the Only Day 3 group also very closely resembled the fish in the

control group with a healthy rate of development and the outline of the body began to become visible. Out of the 30 embryos in the All 3 Days group 21 were determined to be dead because of the bacteria growing inside of the chorions. The embryos that had survived had inflamed stomachs and slight bends in their tails.

The zebrafish were placed into the incubator overnight and after 48 hpf they were then examined again. There were no more deaths found in the control group and showed normal development. They all had normal body parts, normal heart beats, and did not have the slight bend in the tail like the embryos that were exposed to caffeine. In the Only Day 1 group there was also no more deaths, but the abnormalities were only more exaggerated this time with inflamed stomachs and quick sudden movements. The tails of the embryos were still bundled up inside of the chorion. None of the embryos had died in the Only Day 2 group, but the chorions were visibly weak. Only one of the embryos had died in the Only Day 3 group. Some of the embryos that were alive were were beginning to break out of the chorion and they were very developed. None of the embryos in the All 3 Days group had died over the 48 hpf period. These embryos appeared to have a slow rate of development. The embryos eyes were clear. Compared to the control group the tails were undersized and their stomachs were enlarged.

The final observations were obtained 72 hpf. In the control group there were no deaths. A large majority of the embryos had hatched or were very close to hatching. Their heartbeats were steady and normal and all body parts were at a normal proportion. These zebrafish looked very different compared to the embryos exposed to caffeine. All of the fish exposed to caffeine on only day 1 were also alive. Some of the zebrafish had hatched and there was a distinct bend in the tip of the tail. One of the fish that had hatched only had one fin causing it to swim in circles. The embryos that had not yet hatched were visibly shaking in their chorions. All of the zebrafish had noticeably enlarged eyes and a wrinkly, weak chorion. Only one of the fish had died out of the fish exposed to caffeine only on day 2. The zebrafish that were alive appeared very jittery. They also had a noticeably weak chorion, abnormally inflamed stomachs, slight tail bends, and very slow heartbeats. Fourteen of the embryos in the Only Day 3 group had died. The fish that had hatched had very bent tails and very slow heartbeats; 28-33 bpm. Out of the zebrafish exposed to caffeine on all 3 days 2 of them had died. The embryos that did not yet hatch had frequent spasms inside of the chorion. They also had very large stomachs compared to their underdeveloped bodies. They had tiny eyes, very little cell differentiation, their bodies were shaped like an ‘S’, and they were barely alive with a weak heartbeat.

The aim of the experiment was to find which period of exposure to 1.00 mg/mL of caffeine was most crucial to the development of the zebrafish. The quantitative data found by the death rate was not significant between any of the groups including the control group. The qualitative data recorded, however, showed that high concentrations of caffeine in the early stages of development causes deformities and can even cause death. The data showed that all stages of development are of equal importance.

Discussion

Our results agree with previous research on the effects of high concentrations of caffeine on zebrafish (Brunning, 2014). It was known that the high concentration of caffeine would have a very significant impact on the development and survival rate of the embryos and our data shows that. The most significant qualitative data that was recorded in this experiment include the bent tail end, the abnormally enlarged stomachs, weak chorions, and the very slow heartbeats at about 28-33 bpm. These deformities were found in all of the zebrafish that were exposed to caffeine giving this data validity even though our quantitative data was lacking significance. The results show that all periods of development are crucial and the caffeine will cause deformities regardless of the day the embryo is exposed to the high concentration of caffeine. This contradicts my hypothesis that the exposure to caffeine in the beginning stages of development will have the largest impact on the development of the zebrafish embryo.

The results suggest that all stages of development are crucial. The importance of each stage means that it is critical that pregnant women limit their intake of caffeine throughout their entire pregnancy because it could cause deformities or slower development in the child.

There may have been human error in the beginning of the research when identifying whether or not the embryos were fertilized. By misidentifying embryos as fertilized in the beginning could have caused a higher death rate. Limitations set by the microscope could have caused signals that embryos were or were not fertilized to be missed.

To expand upon this research an experiment should be done that tests to see if the same result will occur if a lower concentration of caffeine is used. Once again expose the zebrafish at different intervals of time, but with a lower concentration the death rate and abnormalities should greatly decrease which could help to differentiate between which stage of development is the most critical.

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