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The Effects of Caffeine on the Development of Zebrafish Embryos

ABSTRACT

An experiment was performed to identify the effects of having too much caffeine in the human body. This is important because a lot of people drink caffeine and do not know the long term effects of being exposed to it. Zebrafish embryos were used as an experimental species and were exposed to three different concentrations of caffeine (0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL) to see the effects. The results of this experiment were that most of the embryos exposed to the lower concentrations of caffeine developed deformities such as swollen yolk sacs, curved spines, underdeveloped bodies, and few deaths occured. However, all of the embryos exposed to 1.0 mg/mL concentration died. These results were significant because they showed that too much caffeine could harm a developing embryo, or even kill it according to observations. This experiment was done over 96 hours and consisted of 10 embryos per well, three experimental concentrations, and three control groups. Others who could have performed this experiment might have used a different time frame, number of embryos, and number of experimental and control groups.

INTRODUCTION

The purpose of this lab is to find out how a chemical, like caffeine, affects the biological development of embryos. This investigation is conducted to find out how dangerous caffeine

can be. Zebrafish are used for this for several reasons. First of all, they mature rapidly and are easy to obtain. They are fertilized externally and female zebrafish can lay up to 200 eggs a week. Secondly, their embryos are translucent and this allows people to keep track of them as they mature. Lastly, they have a similar number of genes as humans do (*Zebrafish facts,* n.d). This makes them ideal to study and use to compare with humans. The hypothesis of this experiment is if zebrafish embryos are subjected to different concentrations of caffeine, then some of the fish will develop deformities or will die.

Through prior research to this experiment, it is found that when embryos were exposed to various chemicals, such as caffeine, they develop with deformities. According to one scientific report talking about how caffeine affects the embryonic development of a chicken, exposure resulted in, "...neural tube closure and....disorder of serotonergic system development…"(Li,X. et. al, 2012). Furthermore, when pregnant mice were exposed to caffeine to see the effects on the developing mouse embryo inside, it was found that the embryos had, "both short-term effects on cardiac development and long-term effects on cardiac function," (Wendler, Busovsky-McNeal, Rivkees, 2008). All of this research contributed to the development of the hypothesis of what would happen when zebrafish embryos are exposed to caffeine. Zebrafish embryos are exposed to different levels of caffeine in order to learn how this chemical affects the embryos. The zebrafish are an adequate match to human embryos because zebrafish have 70% of the same genes as humans. In addition, there is a plethora of eggs that are laid by zebrafish and are therefore dispensable. Since zebrafish embryos are mostly transparent, scientists are able to see the effects of different chemicals (Burke, 2016). Research of this is important to know because it proves that certain chemicals are dangerous to unborn human embryos.

MATERIALS

Stock concentrations, zebrafish, plates, and media solution were provided from the UW Milwaukee's Wisconsin Inquiry Based Scientist-Teacher Education Program, funded by Part of NIH Science Education Partnership Program.

- 0.05, 0.25, 1.0 mg/mL of caffeine concentration in 3 different beakers
- Beaker for dead embryos and liquid disposal
- Sharpie for labeling wells and plates
- Instant Ocean/Embryo Media Solution in beaker
- Large bore transfer pipettes, minimum bore 1.5 mm for transferring eggs to observation container and manipulating them in the container
- Small bore transfer pipettes for removing liquid concentrations
- 12-well plate for storing the fish embryos
- 28.5 degrees Celsius incubator to leave fish in overnight
- Depression slide with coverslip to view fish
- Dissecting and compound microscope to view fish
- Latex gloves for safety
- Goggles for safety

METHODS

Prior to the experiment, the embryos were delivered/fertilized at 9:00am, then rinsed in new embryo solution and placed in the incubator at 28.5 degrees Celsius.

1. DAY ONE
    a. Obtained rinsed embryos
    b. Labeled plates with name and concentrations using a Sharpie
    c. Filled one well of the plate with 1mL of Instant Ocean/Embryo Media solution using the disposable pipette. Filled remaining wells with appropriate caffeine stock solutions
    d. Divided the embryos so there were approximately 10 embryos in each well. Labeled on data sheet
    e. Observed embryos under dissecting microscope and recorded observations
    f. Recorded exact number of live embryos, and discarded dead embryos
    g. Placed each plate in the 28.5 degree Celsius incubator overnight
2. DAY TWO
    a. Removed plate from incubator
    b. Removed dead embryos from the plate using the disposable pipette. Placed dead embryos into the waste beaker
    c. Counted remaining embryos and hatched fish and recorded on data sheet
    d. Removed and replaced caffeine and control solutions using a clean pipette
    e. Placed plate with embryos under dissecting microscope and recorded observations on data sheet. Described any developmental marker or abnormalities. Repeated for all concentrations

f. Removed 1-2 embryos and placed them on the depression slide with slip cover. Placed slide under compound microscope. Observed the embryos and recorded appearance and health. Repeated for all concentrations
g. Returned embryos to their well in the plate
h. Returned plate to to the incubator

3. DAY THREE
    a. Repeated Day Two work and recorded all data
4. DAY FOUR-OPTIONAL
    a. Repeated Day Two work and recorded all data
    b. Placed all embryos and fish in waste container for euthanization by bleach at a later time
    c. Rinsed out each well with water to be sure everything was removed
    d. Cleaned up all materials and disposed of them properly

This method was chosen to appropriately examine the effects of caffeine on zebrafish embryos.

SAFETY PROCEDURE

Safety precautions used included using safety goggles, gloves and tying back hair. This was to avoid getting chemicals in contact with skin or eyes. After observing the zebrafish, the tables were cleaned and all materials were disposed of properly. The pipettes were thrown away and used solutions were put into a beaker with the dead embryos. After the experiment, the live zebrafish were put into the beaker with the dead embryos and would later be euthanized in a bleach solution.

HOW RESULTS WERE MEASURED

The effects of the chemical used (caffeine) was measured by whether the zebrafish had deformities when hatched, as well as if deaths occurred or not. Dissecting and compound microscopes were used to see the effects more in depth. Some of the methods for quantitative analysis included having the heart rate measurement of a control zebrafish embryo versus various experimental embryos to determine if any of the embryos had a higher or lower heart rate due to the chemical they were exposed to. The embryos were also counted each day of experimentation to determine the amount alive, dead, and hatched. This information was then recorded in Table 1 and can be compared to other zebrafish experimentations with the same chemicals to determine if the same results were found.

RESULTS

This experiment was conducted to find out the effects caffeine has on developing embryos, specifically zebrafish. If the zebrafish are subject to caffeine, then they can grow abnormally, especially with curved spines. The zebrafish were put in different wells in a well plate, and were introduced to varying levels of caffeine (0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL) on day 1. The caffeine was the independent variable. There were 3 different wells of control fish, each put in a solution of only instant ocean. The effects on the fish, due to caffeine, were the dependent variable. The control was used in order to note the differences between the control and the fish in the caffeine solution. On the last day of the experiment, both the 0.05 mg/mL and 0.25 mg/mL had 9 hatched eggs, and ten were observed to be alive. This shows the underdevelopment of the fish, because all of the control fish had been hatched already. However, the 1.0 mg/mL of caffeine had 0 alive.

Throughout the week, the high concentration of caffeine had placed extreme deficits on the zebrafish that were exposed to 1.0 mg/mL of caffeine. Photos were taken on Day 2 using the compound microscope and depression slides. The zebrafish in Fig. 2, compared with Fig. 1 had an enlarged yolk sac, smaller eyes, and a curved tail. The effects are quite extreme and shows how much caffeine can affect developing embryos. In addition, the heartbeats of both the control group and the 0.25 mg/mL experimental group were measured on the second day. The heart rate of the control group was 152 bpm and the heart rate of the caffeine group was 180 bpm. These results show that caffeine drastically increases the heart rate of organisms. In addition to these deformities, many of the zebrafish died. Graph 1 shows the number of zebrafish living up to 96 hours after fertilization. The embryos exposed to 1.0 mg/mL had the least amount of embryos alive after 96 hours. There were none alive, and this could be due to the high concentration of caffeine that they were exposed to. Surprisingly, the control group had the second least amount of zebrafish left. This might be due to outside factors such as transferring new solution into their well. The other experimental groups had zero losses, however, the zebrafish in these wells were underdeveloped and had deformities compared to the control. All of these results show how caffeine could negatively affect humans, based on how underdeveloped and deformed the embryos ended up being and how higher concentrations of caffeine killed them. The results also support the initial hypothesis that zebrafish embryos exposed to caffeine would end up deformed.

STATISTICAL ANALYSIS

A Chi-test of independence was performed to examine the relation between exposure and death of zebrafish. The relation between these variables was not statistically significant with a

p-value of 0.1253 with a 95% confidence interval. This supports the null hypothesis that there is no relationship between the death of zebrafish and the exposure to caffeine. However, caffeine does cause deformities to experimental groups versus the control group and this can be confirmed due to physical observations. In addition, using larger sample sizes may make this value more accurate.

Figure 1

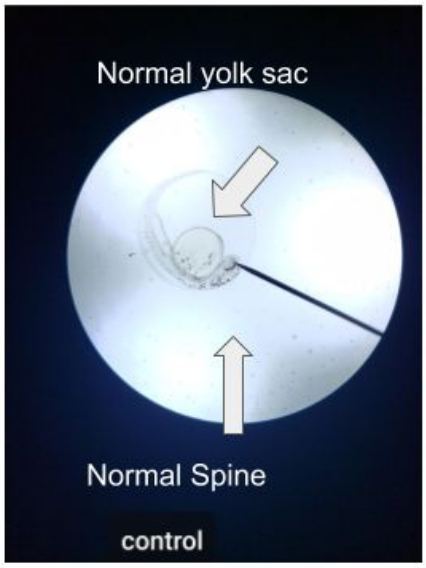


Figure 2

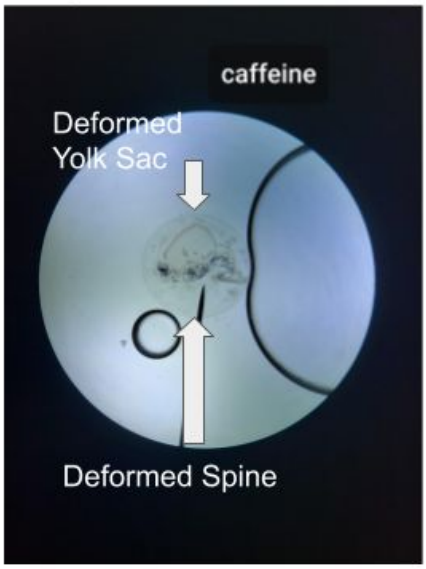
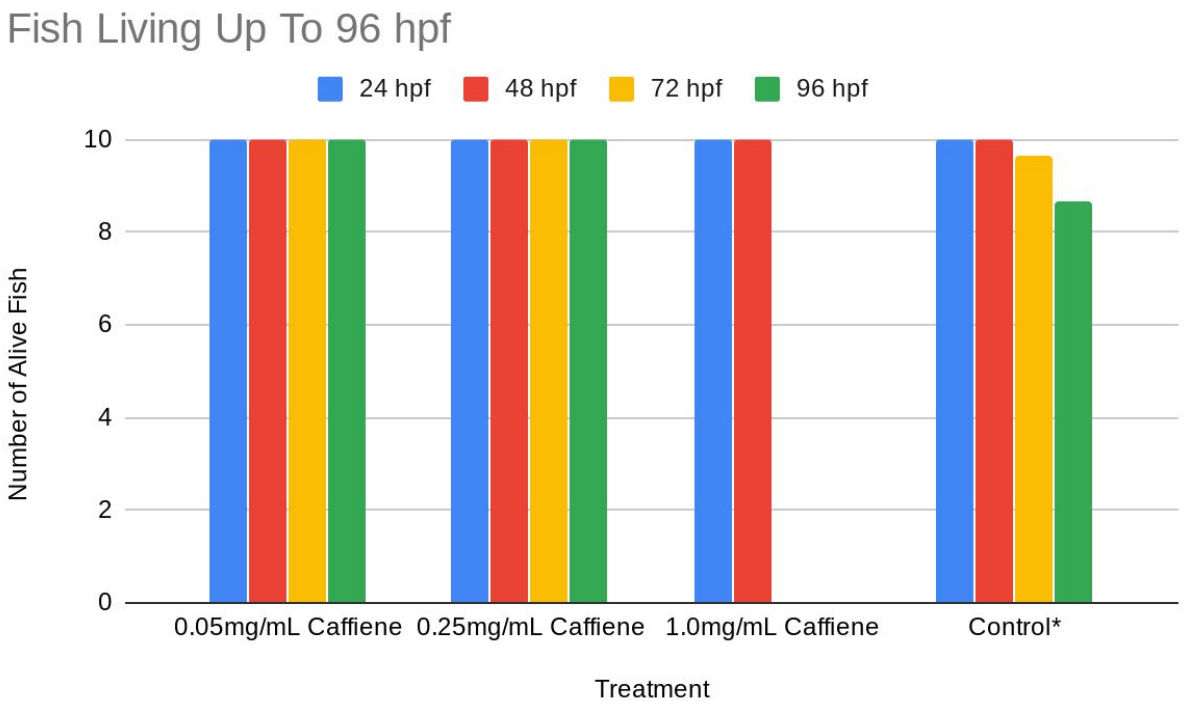


Figure 1: The control fish were not subject to any chemicals and developed at a normal rate. They set the standard for what zebrafish should look like.
Figure 2: The fish subjected to caffeine developed with deformities. They had crooked spines and swollen yolk sacs.



Graph 1: The fish in the highest concentration of caffeine were all dead after 72 hpf*. Some were lost in the control, the control was the average of three wells.

*Hpf is hours post fertilization

Treatment	24 hpf	48 hpf	72 hpf	96 hpf
0.05mg/mL Caffeine	10	10	10	10
0.25mg/mL Caffeine	10	10	10	10
1.0mg/mL Caffeine	10	10	0	0
Control*	10	10	9.67	8.67
*Average of all 3 Controls				

Table 1: Zebrafish embryos in the highest concentration of caffeine were all dead by 96 hpf. Zebrafish embryos in lesser concentrations survived through the experiment. The control group lost some embryos.

DISCUSSION

The results of this experiment show the negative effects on zebrafish embryos. The patterns found in the qualitative data show that all of the experimental groups showed various levels of deformities, where the severity increased when the concentration increased. These deformities include all of the experimental groups having underdeveloped bodies, curved spines, enlarged eyes, and swollen yolk sacs. The quantitative patterns show that as the concentration of caffeine increased the severity of deformities increased as well. This is a positive correlation that shows when more caffeine is present, the worse the deformities are. This pattern is represented by how all the zebrafish were alive in the lower concentrations of caffeine, but were all dead by hour 96 after fertilization in the highest concentration. This supports the hypothesis that caffeine would have negative effects on zebrafish embryos and could cause deformities or death.

Human error could account for some inconsistencies in the data. For example, some embryos could have been disabled or malformed in transfer. Also, pipettes could have been mixed up, and used for several different concentrations, which would alter the results. Miscounting the embryos could have been an issue as well. These results show that caffeine can and does affect developing embryos. This corresponds with the discoveries that Li,X. et. al had found in their experiments with chickens, as well as with the mice that Wendler, Busovsky-McNeal, Rivkees had experimented on. These results provide information on the damages that caffeine can do to developing embryos. More than ever, people are ingesting many forms of caffeine, which is shown to be harmful to developing embryos. Further research could

be done on this topic such as performing the experiment again to verify the consistency of the results and using larger sample sizes to achieve this.

Bibliography

Gluckman, P. D., Hanson, M. A., Spencer, H. G., & Bateson, P. (2005, April 7). Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1602053/.

Li, X. D., He, R. R., Qin, Y., Tsoi, B., Li, Y. F., Ma, Z. L., ... & Kurihara, H. (2012). Caffeine interferes embryonic development through over-stimulating serotonergic system in chicken embryo. *Food and chemical toxicology*, *50*(6), 1848-1853.

Merle/Geisler. (n.d.). HEALTH. Retrieved from http://www.zf-health.org/information /factsheet.html.

Wendler, C. C., Busovsky-McNeal, M., Ghatpande, S., Kalinowski, A., Russell, K. S., & Rivkees, S. A. (2009, April). Embryonic caffeine exposure induces adverse effects in adulthood. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2660649/.

Why Use Zebrafish to Study Human Diseases? (2016, October 14). Retrieved from https://irp.nih.gov/blog/post/2016/08/why-use-zebrafish-to-study-human-diseases.