The Effects of Varying Amounts of Nicotine on the Development of Zebrafish Eggs

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ABSTRACT

The purpose of the experiment was to see how various amounts of nicotine can affect the human body by using zebrafish as a model organism. By exposing zebrafish embryos to different concentrations of nicotine and comparing the changes over the course of five days, observations can be made, of how the zebrafish react, and possibly showing how human fetuses may be impacted by nicotine when exposed in the womb. Zebrafish were used because their nervous systems are similar to a human's. Because of this, inferences that nicotine will have similar effects on the human body can be made. This is an important topic to research because prenatal exposure to nicotine doubles the chances that the baby will either be born prematurely and/or weigh less than 5 ½ pounds at birth. It also doubles the chances of a miscarriage. It was seen in the zebrafish that were exposed to nicotine while developing that the higher the concentration of nicotine, the more deformities and deaths occurred.

INTRODUCTION

Zebrafish have been known for being genetically similar to humans. According to Kurt Svoboda from the University of Wisconsin Milwaukee, zebrafish are vertebrates and have similar embryos, nervous systems and behaviors. "As vertebrates, zebrafish share developmental, anatomical, and physiological characteristics as mammals including humans." (Kurt Svoboda, 2015, p.6). Their embryos are important because they develop rapidly and externally. This means the scientists observing can see the eggs clearly through a microscope. The zebrafish have "simple" nervous and "simple" behaviors. The nervous system responds easily and rapidly. The behaviors are as simple as movement or responding to touch. A key aspect that zebrafish share with humans is that they have nicotinic acetylcholine receptors or nAChRs. This means that if a neuron expresses nAChRs, nicotine exposure would activate that neuron and would enter the cell through the receptor. In simpler terms, this means that zebrafish have cells like ours that can recognize when nicotine is present in the cell.

Nicotine is an addictive substance found commonly in cigarettes. Nicotine is a nitrogen-containing chemical or alkaloid made by many plants, and also can be synthesized. Nicotine has many negative effects on the human body. It mainly affects the brain, heart, arteries, eyes, metabolism, reproductive system, and bones. Nicotine disturbs the neurotransmitters, which causes chemical changes in the brain. According to *Medical News Today*, side effects that are caused by nicotine include dizziness, lightheadedness, sleep

disturbance, and tremors. Nicotine increases heart rate and blood pressure. This causes your body to work harder to move oxygen and blood cells that need it. In the long term, this can cause heart attacks and strokes. Nicotine also affects the eyes, in that it stops the production of pigments that help to see at night. The release of adrenaline, triggered by nicotine decreases peripheral vision. Overall, nicotine promotes the degeneration of the eyes. The effects can also be seen in metabolism, as it decreases people's' endurance. Nicotine affects the reproductive system by increase the chance of infertility and miscarriage. Over time, nicotine weakens the bones, and poses more of a risk of breaks and fractures.

Zebrafish, scientifical known as *Danio rerio* was exposed to various amounts of diluted nicotine solutions over the course of 120 hours (5 days). Throughout this process, data was collected every 24 hours on embryonic development and hatch rate. After data was collected solutions were changed out for fresh nicotine solutions. The solution in each well was kept the same throughout the experiment.

The hypothesis for the experiment was, if the zebrafish are exposed to increased amounts of nicotine, then the death rate will increase because of the effects that will take place on the embryos. This study was performed to find out the effects of nicotine on zebrafish, which could be used to form a hypothesis about how nicotine affects humans.

MATERIALS AND METHODS

For this experiment, a 3x4 well plate with four wells was used for containing eggs and solution. One large bore and one fine bore transfer pipette was used to remove the remaining fluids/dead eggs, two medium sized beakers were used, one as a container to dispose of old solutions, and another to place eggshells and dead eggs/fish. Diluted nicotine solutions: 0.0 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2mg/mL were used to experiment the effects of different amounts of nicotine on zebrafish eggs. A stereoscope was used for examining changes in the eggs. Examination gloves were used to protect hands from getting nicotine on skin. Masking tape was used for labelling wells with numbers, solutions and group members names, and a camera was used for taking pictures through the stereoscope to document the changes of the eggs. Lastly, writing utensils and a notebook were used for recording all collected data, and an incubator, set at 28.5 degrees Celsius was used for maintaining the optimum temperature for developing zebrafish eggs. All materials were provided by the Wisconsin Inquiry-based Scientist-Teacher Education Partnership (WInSTEP) Program, which is part of the NIH Science Education Partnership Award (SEPA) Program administration by the University of Wisconsin-Milwaukee and the Children's Environmental Health Sciences Core Center.

To start the zebrafish experiment, and either support or reject the hypothesis, a spot plate, or well plate was labeled with assigned nicotine solutions of 0.0 mg/ mL, 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL. (The control was the well with 0.0 mg/mL, the independent variables

were the nicotine solutions, the dependent variable was the mortality rate. The controlled variables were the incubator temperature, amount of solution put in the wells, and the time of incubation). Ten zebrafish eggs were carefully put into their four assigned wells. The residual solution from embryo transfer was taken out and replaced with 1 mL of nicotine solution. Examination gloves were used for safety when handling the solution. It was important that the solutions were not handled with bare hands. The well dish was placed into a 28.5 degree Celsius incubator overnight, 24 hours. After 24 hours, each well was examined under a stereoscope and any zebrafish that have hatched, died, or any movement was recorded. Zebrafish were designated as deceased if they had no heartbeat. They were also designated by the color of the zebrafish. If they were solid black, that meant they were deceased. Any deformities or differences between the zebrafish in different wells were recorded as well. After observations were made, solutions were replaced using the fine tip transfer pipets, then the well plate was placed back in the 28.5 degree Celsius incubator for another 24 hours. Over the next two days, the process of changing the solutions and observing was repeated. On day five the final results were recorded and the dead eggs were disposed of and living were placed in an aquarium. Chi square statistical analysis was performed to determine if zebrafish mortality rate was independent of nicotine concentration.

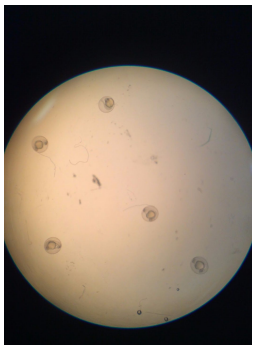
RESULTS

The purpose of this experiment was to prove the hypothesis of, if zebrafish are exposed to increasing amounts of nicotine, than the mortality rate will increase as well. Our independent variable was the varying nicotine solutions, making the dependent variable the mortality rate of the embryos. The controlled variables were the amount of solution in each well, the incubation temperature, and time of incubation. When the zebrafish were exposed to higher concentration of nicotine, the mortality rate increase. A Chi Square Analysis was conducted, and the chi square value was 16.97. Using 3 degrees of freedom and a critical value of 7.82, this lead to the rejection of the null hypothesis. This means that the results were not by chance.

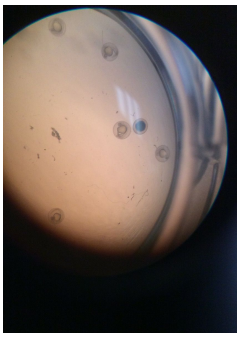
Day 1: On the first day of the experiment, the eggs were a clear color where the organs were visible when under a microscope. The eggs were extremely small, almost unable to be seen by the naked eye. No differences were visible between the control and the experimental.

Day 2: After the first 24 hours of the zebrafish eggs being in varying amounts of nicotine solution, the eggs had undergone changes. The ten eggs in well 0.0 mg/mL were alive. Little movement was noted and the color remained clear. There was no movement in wells 0.05 mg/mL through 0.2 mg/mL and the color was a clearish yellow. In wells 0.05 mg/mL and 0.1 mg/mL one egg died. In well 0.2 mg/mL, two eggs died. No eggs hatched.

Well 1 (0.0 mg/mL)
Well 1 shows how the eggs looked after being the solution for 24 hours. Notice none have hatched and they are only slightly developed.

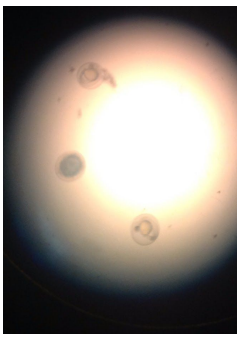


Well 2 (0.05 mg/mL)
In well 2, this is what they looked like after 24 of being in their solution. Small developmental changes were seen, such as slight movement inside the egg.

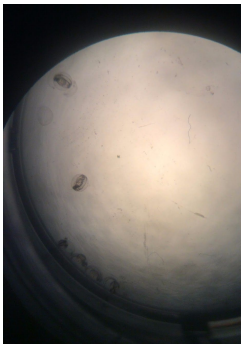


Well 3 (0.1 mg/mL)
In well 3, a dead egg was found under the scope. The dead eggs were identified by a solid black circle in the egg.

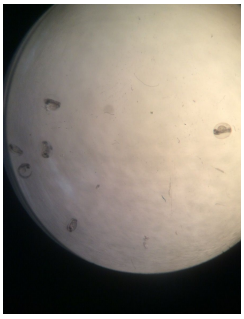
Well 4 (0.2 mg/mL)
This also shows the difference in appearance between live and dead eggs. The live egg is in the early stages of development.



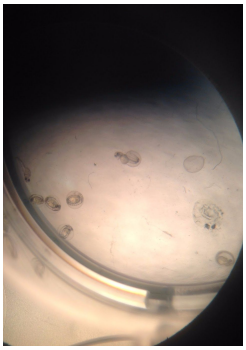
Day 3: The eggs have now been in the solution for 48 hours. Four of the zebrafish in the control, well 0.0 mg/mL, hatched. One of the zebrafish died, there was nine alive. There was very little movement and the color was clear where the organs were visible under the microscope. There were no zebrafish that hatched in well 0.05 mg/mL, though all nine eggs survived. Two zebrafish hatched in well 0.1mg/mL and all nine eggs survived. In well 0.2mg/mL, one egg hatched and all eight survived.



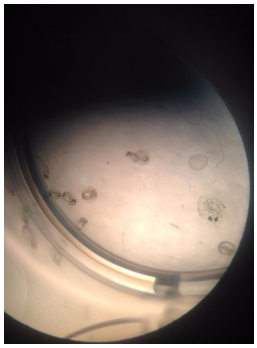
Well 1 (0.0 mg/mL)
They had little movement this day.
A clear heartbeat could almost be seen.
After cleaning out the solutions a better
image of the fish inside the embryo
was able to be seen.



Well 2 (0.05 mg/mL)
One fish has died. Development could be
seen in the remaining eggs. Notice that some
of the zebrafish are beginning to develop
tails.

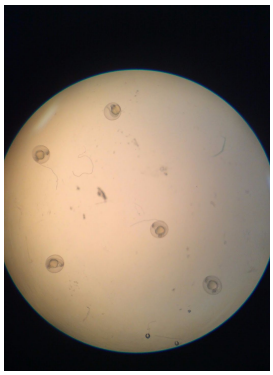


Well 3 (0.1 mg/mL)
This is how the fish looked after
48 hours of being in the solution. This image shows
that many of the zebrafish eggs are developing.



Well 4 (0.2 mg/mL)
There was one egg that had hatched today.

<u>Day 4</u>: During the fourth day of observation, the zebrafish eggs had experienced major changes. In Well 0.0 mg/mL, all nine eggs survived one more hatched. This added up to a total of 5 zebrafish hatched. In Well 0.05 mg/mL, all of the eggs hatched, and one died. A total of eight hatched zebrafish eggs are alive in well 0.05 mg/mL. In well 0.1mg/mL, five more eggs hatched, making a total of seven eggs. One died, totaling to only eight eggs in the well. Lastly in well 0.2 mg/mL, five more hatched, totaling six hatched eggs. Only two remain living. In wells 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL there was little movement and little black spots were visible. In well 0.0 mg/mL, there was lots of movement and the eggs were clear with black spots. The rate of living zebrafish eggs can be seen in Figure 1 below.



Well 1 (0.0 mg/mL)
This well shows how the zebrafish look under the scope when they have hatched. This day's data was collected The results were that all the eggs had hatched that were alive.

Well 2 (0.05 mg/mL)
This well shows that all but one of the fish had hatched; with a total of nine living fish.

Well 3 (0.1 mg/mL)
Every fish hatched, however, three didn't survive.

Well 4 (0.2 mg/mL)
Well four shows that all eight of the fish had hatched from the previous day. Only one of the eight survived.

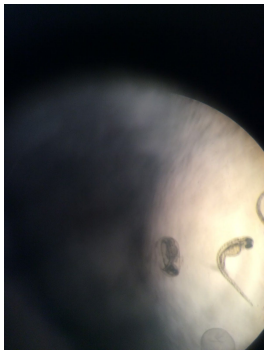
Day 5: After 96 hours of being in the various nicotine solutions, the zebrafish that were alive from the previous day were found dead, except for five in the control. The living zebrafish didn't move much but had very slight movements if closely observed. When the experiment was completed, the remaining living zebrafish were put in a tank to spend the rest of their lives, and the dead ones were disposed of with care. All final results of living and hatched zebrafish eggs are in Table 1 below.

Well 1 (0.0 mg/mL)
There was not much movement in well 1, but
the five remaining live zebrafish still had a clear heartbeat.

Well 2 (0.05 mg/mL)
Well two contained zero living after their 120 hours in observation. They were a light almost translucent gray as they laid in the bottom of the containment.

Well 3 (0.1 mg/mL)
The remaining zebrafish were no longer living after observations after 120 hours. Like well two, the zebrafish were laid in the bottom of the well with an almost translucent gray color to them.

Well 4 (0.2 mg/mL)
After 120 hours, none of the zebrafish had survived. The last of the well 4's zebrafish laid at the bottom with no movement



and no heartbeat, thus the conclusion was that they had died

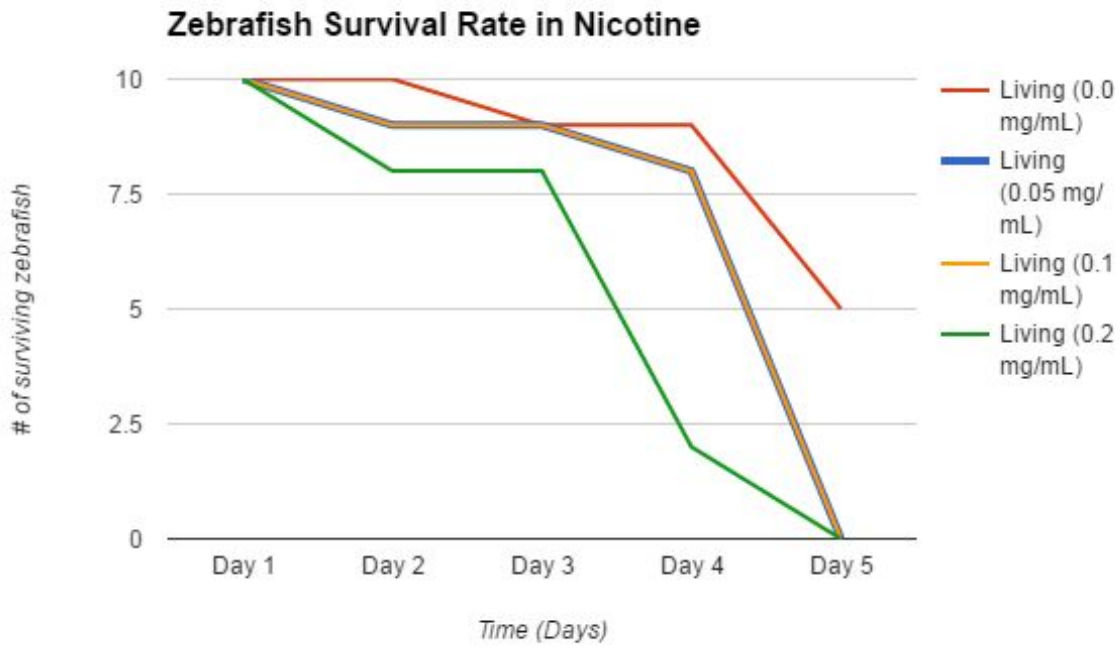


Figure 1: Zebrafish Survival Rate in Nicotine

Figure 1 shows the zebrafish eggs survival rate over the course of the five days. Each well started with 10 living eggs. The lines show the decreased number of eggs each day. Notice that the well with 0.2 mg/mL has the steepest decline, and the only well with remaining live zebrafish is 0.0 mg/mL.

Nicotine Solutions		Day 1	Day 2	Day 3	Day 4	Day 5
0.0 mg/mL	**Living**	10	10	9	5	5
	Hatched	0	0	4	5	0
0.05 mg/mL	**Living**	10	9	9	8	0
	Hatched	0	0	0	9	0
0.1 mg/mL	**Living**	10	9	9	7	0
	Hatched	0	0	2	7	0
0.2 mg/mL	**Living**	10	8	8	2	0
	Hatched	0	0	1	8	0

Table 1: Number of Living and Hatched Eggs in Each Well Each Day
Table 1 shows that the number of living zebrafish eggs in each well each day as well as the number of hatched zebrafish eggs in each well each day.

Chi Square Analysis Tables:
Table 2

Treatment	Live	Dead	Total of Rows
Control	5	5	10
0.05 mg/mL	0	10	10
0.1 mg/mL	0	10	10
0.2 mg/mL	0	10	10
Total of columns	5	35	40

Table 2 above (*Table 2)* shows the end results of the remaining living zebrafish as well as the dead. As seen in the living column the only living zebrafish were in the control well. When it comes to the dead column there were five in the control column and ten in the rest of them, this

means all the zebrafish in the wells other than the control well died sometime during this experiment.

Live

Table 3

Treatment	O	E	(O-E)	(O-E)^2	$\frac{(O-E)^2}{E}$
Control	5	1.25	3.75	14.0625	11.25
0.05 mg/mL Nicotine	0	1.25	-1.25	1.5625	1.25
0.1 mg/mL Nicotine	0	1.25	-1.25	1.5625	1.25
0.2 mg/mL Nicotine	0	1.25	-1.25	1.5625	1.25

Total:15

Table 3 above shows the results when the number of live zebrafish at the end of the experiment was put into the equations.

Dead

Table 4

Treatment	O	E	(O-E)	(O-E)^2	$\frac{(O-E)^2}{E}$
Control	5	8.75	-3.75	14.0625	1.61
0.05 mg/mL Nicotine	10	8.75	1.25	1.5625	.12
0.1 mg/mL Nicotine	10	8.75	1.25	1.5625	.12
0.2 mg/mL Nicotine	10	8.75	1.25	1.5625	.12

Total: 1.97

Table 4 shows the results when the number of dead zebrafish at the end of the experiment was put into the equations.

Chi Square Value: 16.97

The total is the chi square value, which can be used to accept or reject the null hypothesis. In the case of this experiment, the null hypothesis was rejected.

DISCUSSION

The exposure of nicotine to zebrafish embryos can cause heart failure and can double the chances of having developmental deformities. Throughout the experiment, observations supported the original hypothesis of, if zebrafish are exposed to higher amounts of nicotine, then then they will die at a faster rate. It was seen that zebrafish in the higher concentrated solutions had a higher mortality rate. Over time, more zebrafish died in the concentrated solutions that the control solution. Development was clearly being seen. A trend was seen in how many eggs were dying in each well as the experiment continued. The well with the highest level of the nicotine solution, well 0.2 mg/mL, had the highest death rates in the shortest time. The opposite results are shown for the well with the least amount of nicotine. The control well had the most hatched eggs and the least amount of deaths. As concentration of solution increased, then the zebrafish death outcome increased. Over the course of the experiment, observations of deformities/deaths between different wells helped identify what amounts of nicotine affected the zebrafish eggs the most.

In conclusion of the experiment, it was seen that the zebrafish eggs that were exposed to higher concentrations of nicotine typically lead to a higher mortality rate. Possible project errors include not changing the solution at the same time of day each 24 hours, although the time that the solutions were changed were roughly the same time. Any improvements for further experiments of this kind would be to change the solution at the same time and to observe closer to deformities. There were only 40 total zebrafish that were experimented on (10 in each well), so it is possible that the results are not completely accurate, a larger amount of test subjects should be used. The experiment also should be repeated multiple times. Throughout the experiment, observations were made to support the hypothesis, and the Chi square analysis also supported the hypothesis of, if zebrafish are exposed to increasingly higher amounts of nicotine, then the mortality rate will increase as well.

Possible questions for further research could be regarding the change of mass of the zebrafish eggs when exposed to nicotine. If the zebrafish lose weight throughout the experiment, it could mean that they aren't getting enough nutrients and this could also be fatal. Another research question could be how the solutions could affect zebrafish depending if the zebrafish came from captivity or the wild. If the zebrafish are raised in captivity, their immune system

could be impact in a more negative way. If the zebrafish are found or raised in the wild, their immune systems might be stronger against toxins or chemicals.

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