The Deadly Effects of Nicotine on Zebrafish Embryos

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Abstract

Research has shown that nicotine can harm the human body and early fetal development. Research has also shown that zebrafish embryos have similar genes to humans. Therefore if nicotine is present during embryonic development, then it will affect the baby when it is born. The new baby may suffer from birth defects or deformities. The baby may also be at risk of dying at a young age due to nicotine. For this experiment zebrafish embryos were used as a model organism. To be able to do the experiment, a well plate was needed and in each well there were increasing concentrations of nicotine. Well 1 contained 0.0 mg/mL of nicotine, well 2 contained 0.01 mg/mL of nicotine, well 3 contained 0.1 mg/mL of nicotine, and well 4 contained 0.2 mg/mL of nicotine. Over the course of the five days, the mortality rate of the zebrafish and the different deformities/growth issues the fish had were observed. The data showed the difference between the wells and the mortality rates that occurred over the course of the experiment. The fish that were living in the nicotine had a higher mortality rate and suffered from deformities, whereas the zebrafish in the control environment didn't have those issues. Looking at fetal and embryonic mortality rate, it shows how nicotine can harm any living being. This experiment concluded that zebrafish and human embryos alike are negatively affected by nicotine.

Introduction

Many have hypothesized about the correlation between human embryos and nicotine consumption. To prove the relationship, a tropical Southeast Asian fish called zebrafish or *Danio rerio* was used as a model. These fish are a wise model organism to use for many reasons. First of all, they're less expensive to maintain than traditional models (like mice, monkeys, etc.). As embryos, they're nearly transparent. Their transparency allows researchers to identify progress in the development of the fish, and easily examine any deformities. According to ourgenome.org (2014), another reason they're a good model organism is because they have a 70% genome match with humans, and share 84% of genes related to human diseases. In a zebrafish, the major organs share many traits with human organs, and zebrafish embryos developed at a much faster rate than human embryos. Because of these reasons, the zebrafish was an excellent choice for a model organism to test the effects of nicotine on human and zebrafish embryos alike.

Nicotine affects the body in a number of ways. Ucanquit2.org said that when nicotine is consumed, it takes an average of 7 to 15 seconds to arrive to the brain from the blood stream. Once the nicotine is in the brain, it triggers a pleasant feeling from the "reward center" of the brain. After this occurs, adrenaline is released. This increases heart rate, breathing rate, and blood pressure. After continued usage, nicotine's effects can harm the lungs, heart, arteries, and lungs. Over time, heart attack, stroke, and chance of developing a chronic lung disease increases. When nicotine builds up in the body, it can cause a weakened immune system, fatigue, longer healing period, and even cancer. According to Wikström (2007), in a fetus, the nicotine affects the endogenous acetylcholine receptors. These are located in the lungs and brain. Nicotine interferes with neurotransmitters normal functions. This causes neurodevelopmental abnormalities because the timing of neurotrophic actions don't happen on

time. The nicotine that accumulates in the body during pregnancy remains in the body long after giving birth. Wikström (2007) also stated that "Nicotine also accumulates in breast milk, extending the nicotinic exposure to the postnatal period during breastfeeding." This means that the baby would still be consuming nicotine outside of the womb for months.

Consuming nicotine when pregnant can have a number of negative affects on the fetus. Wikström also stated that in 1957, a correlation between pre-term labor in women who smoked was almost doubled what nonsmoking pregnant women experienced. Smoking when pregnant may also cause a heightened risk for preeclampsia. This can happen because of the cardiovascular effects of nicotine including endothelial dysfunction and also raised blood pressure. Sudden Infant Death Syndrome is also a well established side effect of smoking when pregnant along with a reduced birth weight. Utero exposure to nicotine in tobacco can lead to sensory, cognitive, and motor deficits in the toddlers and infants stages. When the child has aged, they're more likely to have a dependency to nicotine.

To investigate the relationship between embryos and nicotine, ten zebrafish embryos were placed into four separate wells. The solutions they were placed in were composed of varying nicotine solutions. The initial hypothesis for this experiment was "if the solution contains more nicotine, than developing zebrafish will have a higher mortality rate, because nicotine causes neurological birth defects. " This hypothesis demonstrates that human embryos would have a very similar, negative reaction to nicotine. This reaction can be harmful to the child, and even result in death.

Materials and Methods

Forty zebrafish embryos were needed to conduct this experiment. Five large bore transfer pipette were used for putting in and taking out the fish embryos. Five small bore transfer pipette was used to remove any residual solution (egg transfer as well as removing nicotine solutions) from each well. The four wells on a 3 x 4 well plate were used to observe the separate environments of fish. The dissecting microscope helped with the process of removing excess liquids and dead zebrafish. A 50 mL beaker was needed so the dead zebrafish embryos and old solutions were able to be removed. The experiment also used four 100 mL beakers which held all the different nicotine solutions. An incubator was set for 28.5 degrees Celsius. Gloves were worn everyday to prevent contamination and nicotine exposure to the skin. All of the 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 administered by the University of Wisconsin-Milwaukee and the Children's Environmental Health Sciences Core Center.

On day 1, the eggs were transferred into the wells. Forty eggs were split evenly into four wells (10 zebrafish eggs per well). Any residual liquid in the wells were removed with the small bore transfer pipette. Increasing levels of nicotine concentration were added into the wells. The concentrations were 0.0 mg/mL of nicotine, 0.05 mg/mL of nicotine, 0.1 mg/mL of nicotine, and 0.2 mg/mL of nicotine. The well tray with the developing zebrafish were then placed into the incubator (28.5°C for 24 hrs).

On day 2 (24 hours, post fertilization), the fish were taken out of the incubator and observed under a dissecting microscope. When examining the fish embryos, the main focus was mortality in fish, how well the fish were developing, and if anything was wrong with the development such as spinal deformities. To determine if the zebrafish were dead or alive, the

focus was looking at the color of the fish. If they were fuzzy looking, gray/white, or black, they were considered dead. A picture was taken through the microscope to help capture the progress of the zebrafish in each of the wells. The large bore transfer pipette was used to remove the dead zebrafish. To remove the old solution, the small bore transfer pipette was used, and the new solutions were added into the well plate using a large bore transfer pipette. The amount of dead zebrafish and the amount of alive/hatched zebrafish were recorded on Table 1 and spinal deformities were recorded on a separate piece of paper. Lastly, the well plate was placed back into the incubator (28.5° C for 24 hours).

On day 3 (48 hours, post fertilization) and day 4 (72 hours, post fertilization) the procedure was the same as day 2. The fish were examined under the microscope, and lastly pictures were taken through the microscope so the process was recorded throughout the five days. The next step was to take the dead zebrafish out of the solution. After that, the old solution was carefully taken out and placed into a 50 mL beaker with the dead fish. Once the removal of the old solution was done, new solutions were equally placed into each well. Finally, the well plate was put back into the incubator over night until the next day. On day 3 and 4, the mortality rate, hatch rate, and the spinal deformities of the zebrafish embryos were recorded. Like the other days, it was recorded using Table 1.

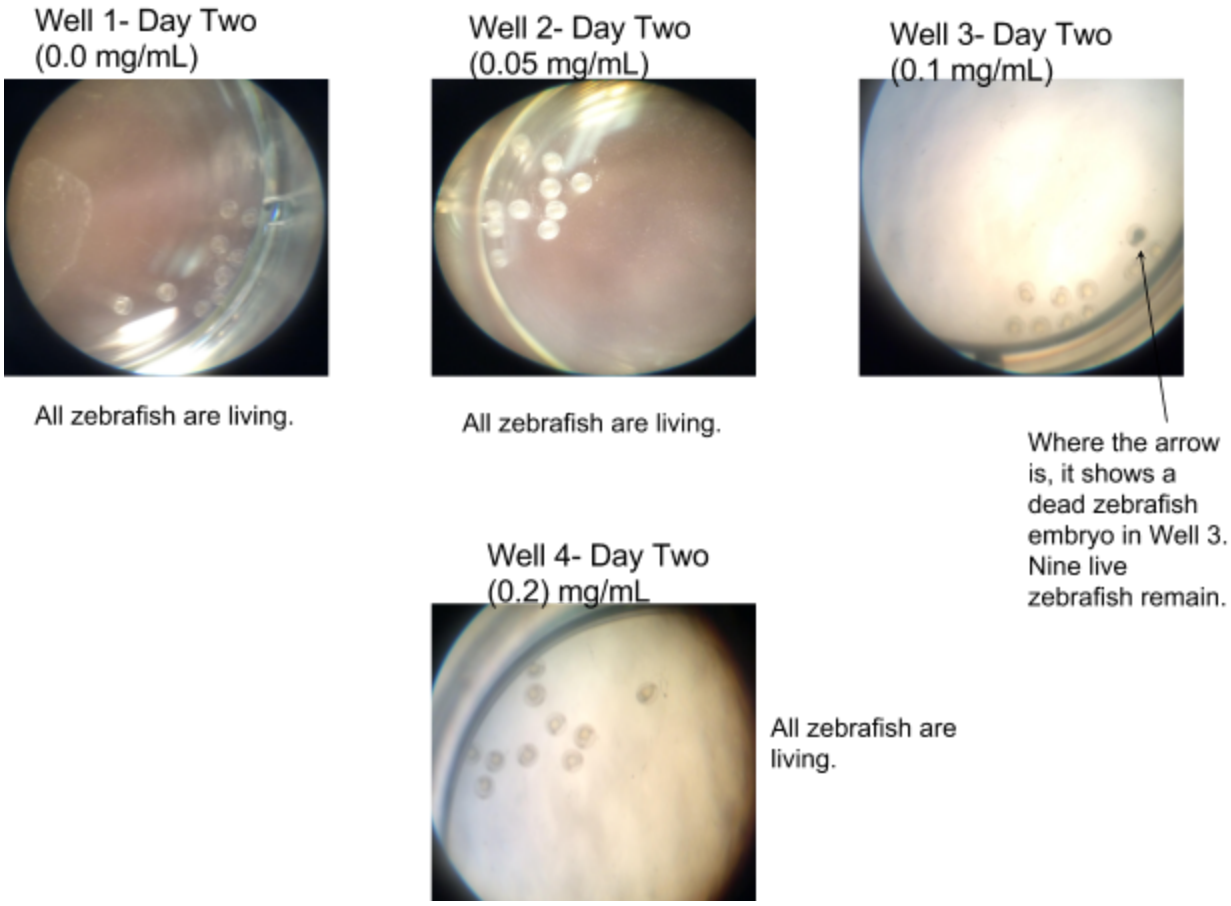
On the last day, the zebrafish were taken out of the incubator for the last time and placed under a dissecting microscope. This time was used to examine all the changes since the previous day. This day was the last time pictures were taken to record the process of this whole experiment. On the last day, all the solutions were removed and the surviving fish were placed in an aquarium to continue their growth/development.

Results

This experiment was setup to observe the impacts of nicotine on the growth of the zebrafish. The experiment showed the change depending on the amount of nicotine in the environment the zebrafish were living in. The independent variable was the amount of nicotine. The dependent variable was the mortality rate in zebrafish. The control variable was the well containing 0.0 mg/mL of nicotine to be able to compare the results to the wells that contained nicotine. The controlled variables were the temperature in the incubator and making sure to keep the zebrafish in their respective wells, the amount of solution added to each well, and size of each well. The overall trend was that as the days passed, 0.1 mg/mL and 0.2 mg/mL of nicotine showed increasing amounts of dead fish and spinal deformities. As concentrations of nicotine increased mortality rate also increased. Based on Figure 5, it showed that in the beginning not many deaths occured, but as time progressed, more zebrafish died.

After the first 24 hours 0.0 mg/mL, 0.05 mg/mL of nicotine, and 0.2 mg/mL of nicotine contained no hatched fish eggs, leaving no dead fish. Contrary to 0.1 mg/mL of nicotine, this well had one loss. The zebrafish looked like small black dots, encased in a clear silicone, comparable to frog eggs. In certain eggs, an average person could see body parts forming. For example, the eyes, body and tail were all visible. The 0.05 mg/mL well had small movements that were detected from the eggs. (These pictures map out the changes in the fish after time. When observing the pictures, make sure to compare the differences between day one and five.)

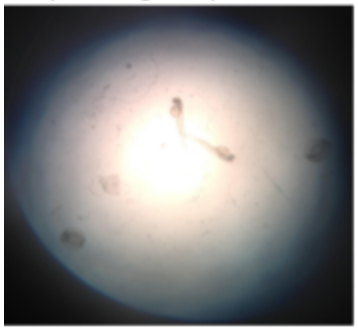
Figure 1- Day Two Photographic Observations



By the next day, (48 hours) each of the four wells had many hatched zebrafish. In the 0.0 mg/mL well, one of the fish died and five were hatched leaving that well with just nine fish embryos. The 0.05 mg/mL and 0.1 mg/mL wells both contained six hatched zebrafish. The 0.05 mg/mL well had a total of ten fish, whereas the 0.1 mg/mL well only had nine fish since one died on day two. Lastly, the 0.2 mg/mL well had four fish hatch and zero dead, leaving the 0.2 mg/mL well with ten remaining fish. The fish that had not yet hatched had long tails and big eyes. The fish were white/clear with black dots on them and some of them also had spinal deformations. One this day, high amounts of movement were detected in the 0.0 mg/mL well, while the other wells had little to no movement.

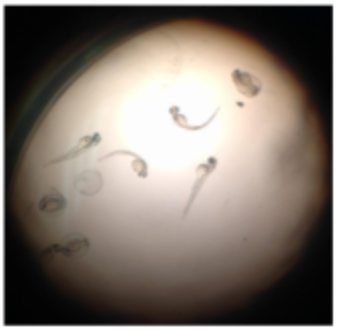
Figure 2- Day Three Photographic Observations

Well 1- Day Three
(0.0 mg/mL)



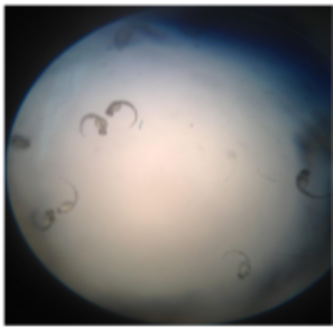
One zebrafish died and five of them hatched. There was a total of nine live zebrafish.

Well 2- Day Three
(0.05 mg/mL)



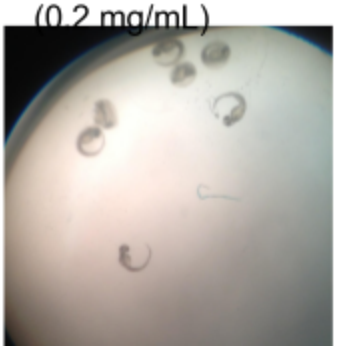
Six zebrafish hatched and there was a total of ten living zebrafish.

Well 3- Day Three
(0.1 mg/mL)



Six zebrafish hatched and there was a total of nine living zebrafish.

Well 4- Day Three
(0.2 mg/mL)

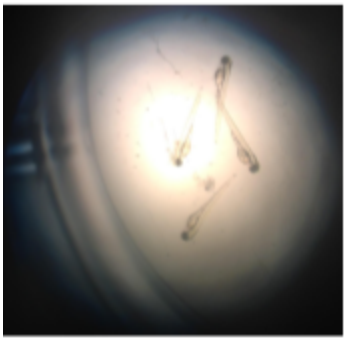


Four zebrafish hatched and there was a total of ten living zebrafish.

After 72 hours, the 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL wells had more than one fish die over the course of 72 hours. In the 0.0 mg/mL well, there were nine fish alive. In the 0.05 mg/mL well there were eight fish living. In the 0.1 mg/mL well there was only one live fish and the 0.2 mg/mL well had five live fish. There were many healthy fish in the 0.0 mg/mL well, but as the nicotine concentration increased there were more spinal deformations. In the 0.1 mg/mL well especially, there were many spinal deformities. There was also a noticeable color difference in the deformed spines versus the healthy fish. The spinal deformities usually were black tails which were generally curled around the fish's body. The healthy fish's tails had a black line where the spine was, and the rest of the tail was translucent. Like the past days, the 0.0 mg/mL well had a high amount of detectable movement.

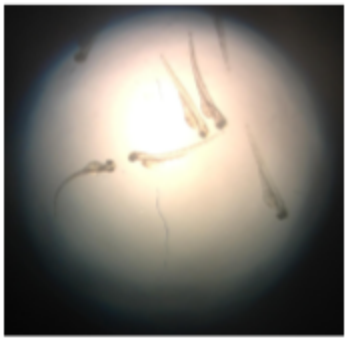
Figure 3- Day Four Photographic Observations

Well 1- Day Four (0.0 mg/mL)



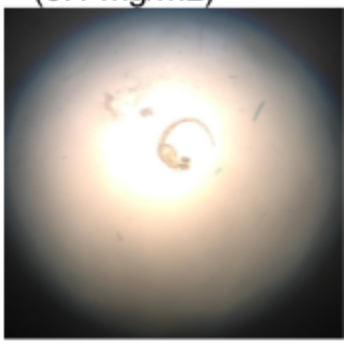
All zebrafish hatched and there was a total of nine live zebrafish.

Well 2- Day Four (0.05 mg/mL)



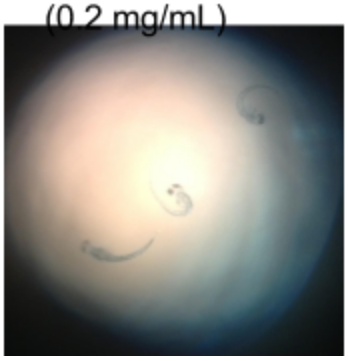
All zebrafish hatched and two died. There was a total of eight zebrafish living.

Well 3- Day Four (0.1 mg/mL)



Only one fish was living from this well and a total of nine died.

Well 4- Day Four (0.2 mg/mL)

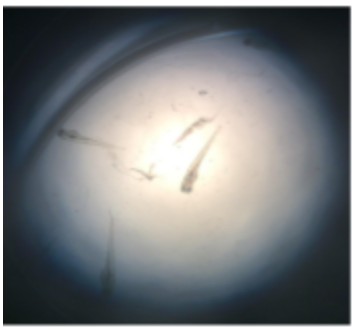


All zebrafish hatched and only five made it. A total of five zebrafish died.

On the last day all of the zebrafish died except for the fish in the 0.0 mg/mL well. In the same well (0.0 mg/mL) there were eight zebrafish living. The zebrafish still alive in the 0.0 mg/mL well seemed fairly healthy. The zebrafish that didn't have to live in nicotine had a higher survival rate.

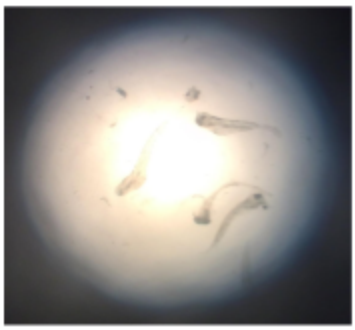
Figure 4- Day Five Photographic Observations

Well 1- Day Five
(0.0 mg/mL)



A total of eight zebrafish remain which means two died in the process of the experiment.

Well 2- Day Five
(0.05 mg/mL)

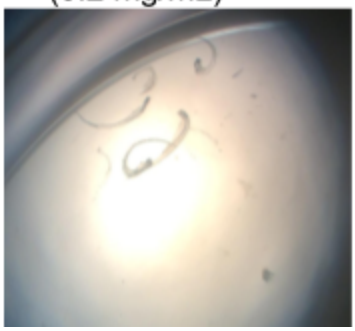


All zebrafish died by the time day five came.

Well 3- Day Five
(0.1 mg/mL)

All zebrafish died by the time day five came.

Well 4- Day Five
(0.2 mg/mL)



All zebrafish died by the time day five came.

The null hypothesis was "If nicotine is added to the solution in the zebrafish wells, then there will be no difference in the fish's development/mortality rate because nicotine isn't a harmful substance." The chi squared table lead us to reject the null hypothesis. The degrees of freedom value was 3, and the critical value for comparison was 7.82. The chi squared value was 30, which is over three times the starting value for a correlation, which showed nicotine had an impact. This shows that the fish in the 0.05 mg/mL, 0.1 mg/mL, and the 0.2 mg/mL wells that have a higher mortality rate wasn't a coincidence. Table 2 and 3 showed the comparison in the total of deaths and the total of living zebrafish using a chi squared tables.

Table 1: Hatched Eggs Versus Live Zebrafish Over the Course of Five Days

Treatment	Well #	# of Starting Fish	24 Hours Post Fertilization	48 Hours Post Fertilization	72 Hours Post Fertilization	94 Hours Post Fertilization
			# Hatched # Live	# Hatched # Live	# Hatched # Live	# Hatched # Live
0.0 mg/mL of nicotine	Well 1	10	0 Hatched 10 Live	5 Hatched 9 Live	9 Hatched 9 Live	8 Hatched 8 Live
0.05 mg/mL of nicotine	Well 2	10	0 Hatched 10 Live	6 Hatched 10 Live	8 Hatched 8 Live	0 Hatched 0 Live
0.1 mg/mL of nicotine	Well 3	10	0 Hatched 9 Live	6 Hatched 9 Live	1 Hatched 1 Live	0 Hatched 0 Live
0.2 mg/mL of nicotine	Well 4	10	0 Hatched 10 Live	4 Hatched 10 Live	5 Hatched 5 Live	0 Hatched 0 Live

Table 1 is the raw data throughout the course of the experiment. The data recorded using this table was the amount of hatched zebrafish eggs, and the amount of zebrafish embryos that were still alive. Based on this data, it was clear that nicotine affected the zebrafish.

Chi-Squared Tables:

Treatment	Live	Dead	Total for Rows
0.0 mg/mL (control)	8(2)	2(8)	10
0.05 mg/mL	0(2)	10(8)	10
0.1 mg/mL	0(2)	10(8)	10
0.2 mg/mL	0(2)	10(8)	10
Total	8	32	40

This table is an aspect of the chi-squared table. This specific table shows all of the data the chi squared table showed how strong the correlation coefficient was in relationship to the zebrafish mortality rate. The calculated expected for the live column (the number in the parenthesis) was calculated by multiplying the total in the live column (8) by the number in "total for rows" in the control row (10) and divided by the overall total in "total for rows" (40). So the equation would be 8x10/40. The same thing would be done with the dead column.

Table 2- The Amount of Live Zebrafish in Each Well (Using a Chi-Squared Table)

Treatment	O	E	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
0.0 mg/mL	8	2	6	36	18
0.05 mg/mL	0	2	-2	4	2
0.1 mg/mL	0	2	-2	4	2
0.2 mg/mL	0	2	-2	4	2
Total					$X^2=24$

Using the Chi-squared table, the amount of living zebrafish were recorded. The main focus of this table is to be able to compare the total of live to the total in Table 3 (the dead).

Table 3- The Amount of Dead Zebrafish in Each Well (Using a Chi-Squared Table)

Treatment	O	E	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
0.0 mg/mL	2	8	-6	36	4.5
0.05 mg/mL	10	8	2	4	0.5
0.1 mg/mL	10	8	2	4	0.5
0.2 mg/mL	10	8	2	4	0.5
Total					$X^2=6$

Table 3 shows the amount of dead over time also using the chi-squared table. Based on table 3, it can be seen that there is a relationship between nicotine and the death of zebrafish.

The graphs below show the comparison between the different wells in two different ways. Figure 5 shows the total amount of deaths throughout the days between the different wells (continuous data). Figure 6 shows the amount still alive throughout the days between the four different wells (continuous data). The graphs legend/ key shows what each of the colors mean. The colors are related to the wells.

Figure 5

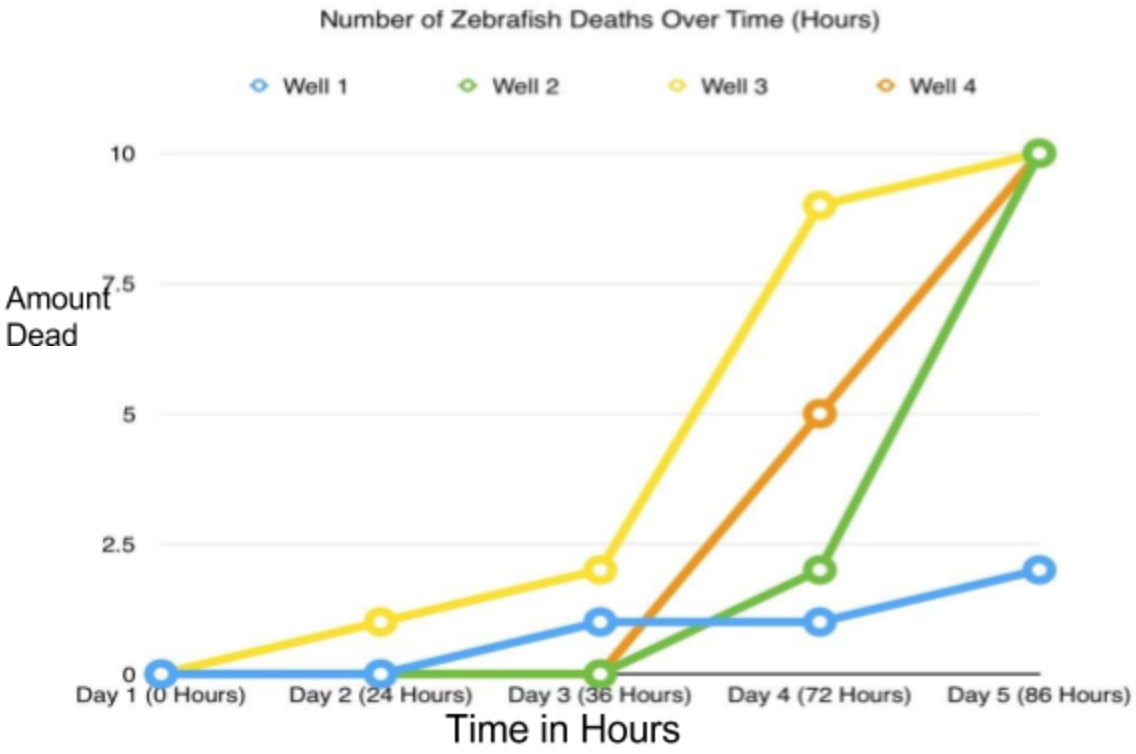
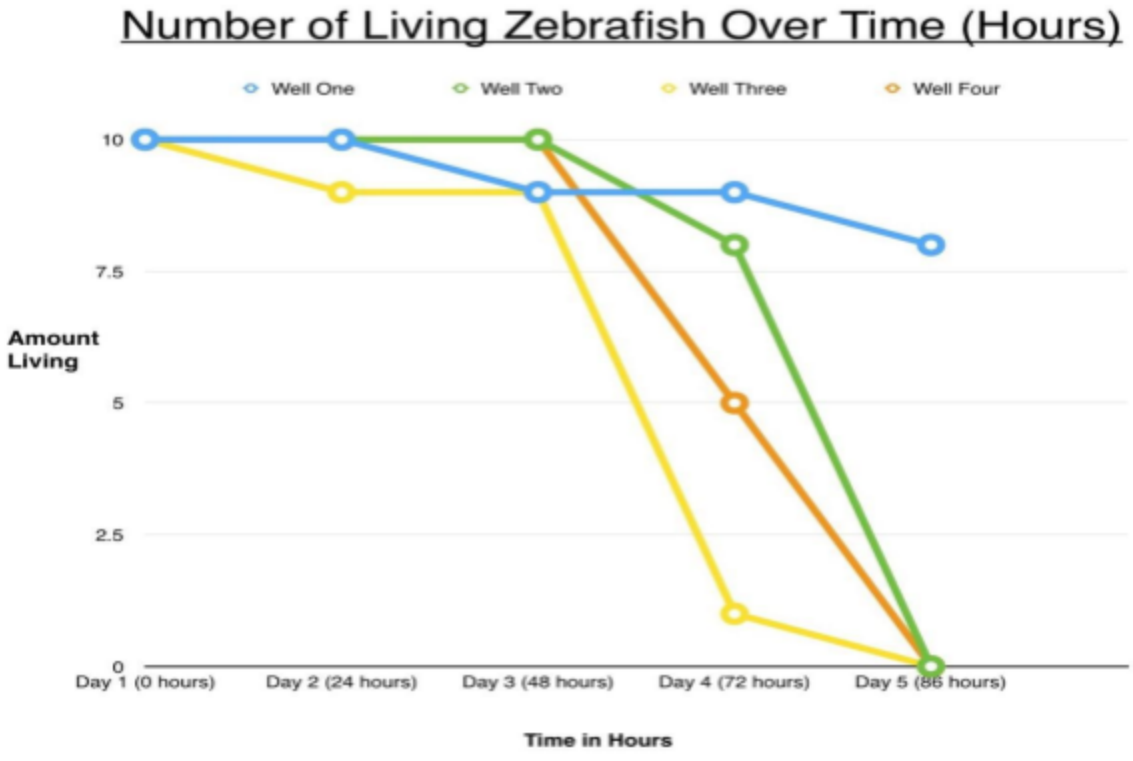


Figure 6



Discussion

With the collective data, it can be proven that higher concentrations of nicotine have a negative correlation on the health of zebrafish, which directly relates to the research collected during the experiment regarding nicotine consumption and how different levels of nicotine can affect human health. Because zebrafish are an accurate model for human research, the effects nicotine left on the zebrafish can be an assumed reaction for what would happen to a human embryo introduced to nicotine. There were also some important trends in the data which show that higher amounts of nicotine in zebrafish result in higher fetal/embryonic mortality rates. There was generally a higher mortality rate and increased amount of spinal deformations in the wells with larger nicotine concentrations. Looking at Figure 6, the correlation is higher than expected. This shows that nicotine is a dangerous substance and in higher quantities kills fish at a faster rate.

Many of the results surrounding the mortality rate and the data collected from the experiment prove the experimental hypothesis. One of the many important results is the Chi Squared analysis. These results are important because they prove whether or not there is a correlation between nicotine levels and zebrafish. Since the chi square value was 30 and the critical value for a correlation was 7.82, it shows that there is a significant correlation between high amounts of nicotine and a higher mortality rate. Embryonic mortality and fetal mortality are in many cases related to the consumption of nicotine in mothers, which also proves the experimental hypothesis. The null hypothesis stated that there was no difference between treatment sections. This also lead to the rejection of the null and acceptance of the experimental hypothesis. In relation, this can be proven the same for human embryos.

In the experiment, there was room for error. One of the possible ways the experiment could've been skewed was time framing. The times weren't always consistent, especially on the last day. On day 5, the solutions were switched almost 5 hours earlier than usual. This could've been improved by switching the solutions within an hour of the usual time every day. Although this was only a small amount of time, it could've lead to inaccurate results surrounding accurate development. Deaths that occurred between those five hours weren't able to be documented which could've thrown data off. Another way data could've been changed was through the usage of gloves as a safety precaution. For the first two days, gloves were not used which could've contaminated the water thus killing fish at a faster rate. To improve this aspect, gloves should be worn from the beginning of the experiment until the end. There was also a possibility that the fish could've been injured when the solutions were being replaced which could've thrown the data off, This experiment should also be tested on a larger scale to confirm the results. A larger sample size could be useful.

In conclusion, the experiment proved very useful and important theories surrounding nicotine usage in pregnant women. The index for correlation between mortality rate in zebrafish and nicotine showed that there was indeed a difference in regular mortality rate in zebrafish and zebrafish introduced to nicotine. The data showed many trends in spinal deformations and developmental issues surrounding the fish in the 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL wells. This shows that human embryos introduced to nicotine would have the same negative results. The data caused an acceptance to the hypothesis and proved the speculations of many scientists throughout the years. It showed that nicotine is indeed harmful, and should be avoided by pregnant women to insure a long and healthy life for the baby. The data clearly shows that nicotine is a fatal substance to human and zebrafish embryos alike and should be avoided by

pregnant women. For further study, which birth defects, such as spinal deformities, or other deformities in the zebrafish were most common?

Citations

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