Effects of Nicotine on Zebrafish Development: Health and Deformities

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Abstract-

Studies have stated that women should not smoke while pregnant, but why is this? The research involved in those studies say that, after looking at trends births by light, medium, and heavy smokers, babies born by heavy smokers have heart defects and a lighter body mass. One of the major chemicals found in cigarettes is nicotine, and as smoking is a pastime favored among many, it was chosen to find the effects of specifically nicotine on the development of a human child. This is a major concern due to the known effects caused by smoking, and the fact that people ignore to the warnings. By demonstrating the effects caused just by increasing concentrations of nicotine, it is hoped to convince those people otherwise.

This experiment, designed to eliminate any risk of contamination from outside sources, isolated four groups of zebrafish and exposed them to various concentrations of nicotine. They were exposed for five days, and had the liquid replaced every 24 hours. Each day, the mortality was recorded and any inconsistencies in their development were observed. Because zebrafish are alike to humans in their DNA, any effects can be modeled for human health. That would prove any effects of a new strain of a disease - or in the case of this experiment, a chemical - on the development of a fetus. However, due to some unprecedented mistakes, the results were insignificant and had no pattern definitively caused only by nicotine.

Introduction-

The following study is an experiment conducted on the embryos of zebrafish, which were exposed to different concentrations of nicotine. It is a known fact that zebrafish are excellent models for the effects of disease, chemicals, and radiation on humans, as zebrafish share 70% of a humans DNA, and include many of a human's internal organs. They are much easier to experiment on than mice, due the natural instincts of population grouping. Whereas mice live in small groups, zebrafish live in schools. Therefore, they allow for a better controlled study (Burke, 2016).

Although there have been studies done on humans, they focus on the effects of smoking while pregnant, as it is rare anybody would have pure nicotine in their household and would therefore be a somewhat unnecessary study. However, this means the effects cannot just be

attributed to nicotine, as the smoke or other chemicals could also play a part in any deformities or mutations in a person's DNA. Therefore, to single out any results of nicotine on the development of a fetus, all the while not risking human lives, zebrafish are used.

In this study, the mortality of these zebrafish, as well as any developmental flaws, were observed. Nicotine was chosen because of the commonly know widespread uses of E-cigs and cigarettes. Smoking while pregnant is a major problem in the world, and being educated about it is important. It was hoped that lead could be used instead for the same reasons, but that was against policy. In this experiment, four groups of ten zebrafish were exposed to differing amounts of nicotine and observed for a series of five days. Due to previous tests, it is known that nicotine causes many problems related to growth deficiencies and hyperactivity.

According to Adam Felman (2018) “When humans, mammals, and most other types of animals are exposed to nicotine, it increases their heart rate, heart muscle oxygen consumption rate, and heart stroke volume”. According to a different source, Wickström (2007) states that “Women using smokeless tobacco have also been shown to give birth earlier than women not using tobacco (mean difference 6.2 days), increasing the risk of preterm birth by a factor of 1.4… Compared with nonusers, the adjusted mean birth weight was reduced in snuff users by 40 g, in light smokers by 172 g, and in moderate-to heavy smokers by 224 g… The association between the risk for Sudden Infant Death Syndrome (SIDS) and maternal smoking during pregnancy is well established and smoking is the strongest risk factor for SIDS…”.

Based upon that knowledge, it can be reasonably hypothesized that, if the zebrafish are exposed to increasing concentrations of nicotine, then the zebrafish in the largest concentration will experience premature birth and have the most casualties and physical deformities because of the risk of SIDS, higher heart rate, and premature birth all caused by nicotine. Throughout this experiment, that will be what is observed and recorded.

Materials and methods-

Materials and Equipment

- (1) Stock solutions of nicotine (0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL Nicotine)
- (5) 100 ml Beakers (one for the deposit of dead embryos and used liquid, four for the different dilutions)
- (1) Sharpie for labeling
- (1) Instant Ocean/Embryo Media Solution
- (5) Large bore transfer pipette
- (5) Fine bore pipette
- (1) Multi-well plates
- (1) 28.5 ºC incubator
- (1) Dissecting microscope
- Data sheet
- methylene blue 0.01%

Notice: When creating your dilutions, make sure you wear gloves and eye protection. Nicotine may cause skin and eye irritation if contact happens. Wear a breathing mask as to not accidentally ingest or inhale nicotine. If any of the prior happens, follow the directions from the SDS, which is conveniently pasted below.

- **Eye Contact**: Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Cold water may be used. Get medical attention.

- **Skin Contact**: In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Cover the irritated skin with an emollient. Cold water may be used. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention immediately.

- **Serious Skin Contact**: Wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. Seek immediate medical attention.

- **Inhalation**: If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

- **Serious Inhalation**: Remove the victim to a safe area as soon as possible. Loosen tight clothing such as a collar, tie, belt or waistband. If breathing is difficult, administer oxygen. If the victim is not breathing, perform mouth-to-mouth resuscitation. Seek medical attention.

- **Ingestion**: If swallowed, do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Loosen tight clothing such as a collar, tie, belt or waistband. Get medical attention immediately.

- **Serious Ingestion**: N/A

First, the multi-well tray was labeled with the different concentrations of nicotine, along with the names of the people in the group and class period. Using the large bore pipette, exactly ten embryos were collected and placed in all wells that were being used in the test. It was determined if the embryos were alive or dead. Those that looked like white rough balls were dead, and needed to be discarded immediately. Once all the fish were sorted out, all the liquid was drained using a fine tipped pipette, and was replaced with 1 ml of the proper stock solutions of nicotine. It is vital for the solutions to correspond with the label given it to avoid confusion. Next, the embryos were observed with the compound microscope. The initial observations of the fish were recorded on the data sheet and later placed into the incubator.

On the second day, development may had started, and the observations were starting to become more important. The first step of day two was to remove any dead embryos from the solution. If left in the tray, they would start to decompose and start a bacterial or fungal infection. The old solution was drained from the wells due to the risk of said bacterial or fungal infection in

the water. The fine tipped pipette was used again to carefully drain only the nicotine solution, being careful as to not grab the embryos. Any applied suction would have broken or injured the egg, resulting in a premature birth. The fresh solution was again into each corresponding well and the embryos were observed. Any developmental markers in the zebrafish were recorded, as well as the survival rate (in fish) and any physical deformities (compared to control). This was done for all concentrations of nicotine. Afterwards, the tray was placed back in the 28.5 °C incubator for 24 hours.

The same steps as day two were done on day three and four, although the zebrafish had started to hatch. Because they were, draining the wells of the solutions had to be done with extreme caution, as to not discard any fish by accident. Although some fish had looked dead, they were just lying on their side and resting. They were not discarded along with any dead fish. On the fourth day, draining became easier, due to the responsiveness of the fish. The had swam away from the suction of the pipette. Of course, that does not mean that the task was not done with utmost care, and an eye was still kept out for any fish in the waste beaker.

On the fifth day, if the experiment was continued, draining was not done, but observations were still recorded. A final column on the data sheet was filled out and computations on the survival rate and observed deformities were recorded. At the end of the day, the fish were either disposed of them or placed in a designated fish tank.

A chi-square analysis was performed on the data to test for statistical significance of data.

Results-

This study has been done to show and explain the effects of nicotine on the development of humans. More specifically, the mortality and birth time of the test subjects, zebrafish. The aforementioned zebrafish are excellent examples for the effects of chemicals on the human body

with the large percentage of shared DNA with humans, at around seventy percent. Before any experimentation, it was hypothesized that zebrafish in the dilution of 0.2 mg/mL would have the most casualties, while those in the control would have nearly all, if not, all fish surviving. The reason this study was done was to show the risks of smoking while pregnant, which is a modern day problem and can be applicable to other's lives.

The design of the experiment was a success. Each of the four wells was filled with one milliliter of solution, each being either 0.0 (control), 0.05, 0.1, and 0.2 mg/mL of nicotine dilutions (independent variable). With the incubation temperature, amount of solution, and size of the wells the same, the experiment held a constant, single changing variable of nicotine concentrations. This was set up in this method to keep accurate records and prove significant results. The results that were recorded was the mortality of zebrafish and the rate at which zebrafish hatched (dependant variable: see Fig. 1 and Fig. 2).

Number of zebrafish embryos alive after exposure to differing dilutions of nicotine as measured over five days

	Initial	After 24 hours	After 48 hours	After 72 hours	After 96 hours
0.0 mg/ml nicotine	10	9	9	9	9
0.05 mg/ml nicotine	10	6	6	6	5
0.1 mg/ml nicotine	10	7	7	7	7
0.2 mg/ml nicotine	10	8	8	7	6

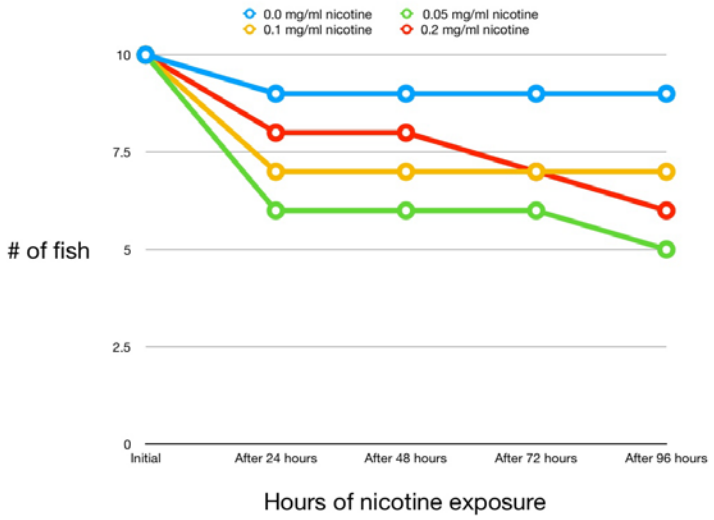

Fig. 1

Figure 1: The number of zebrafish embryos alive after nicotine exposure; a general trend of greater casualties was seen as the concentrations went up. 0.05 and 0.2 mg/mL had the lowest amount of casualties, although were not the two highest concentrations.

Number of zebrafish embryos hatched after exposure to differing dilutions of nicotine as measured over five days

	Initial	After 24 hours	After 48 hours	After 72 hours	After 96 hours
# hatched in 0.0 mg/ml nicotine	0	0	1	9	9
# hatched in 0.05 mg/ml nicotine	0	0	1	6	5
# hatched in 0.1 mg/ml nicotine	0	0	0	6	7
# Hatched in 0.2 mg/ml nicotine	0	0	2	6	6

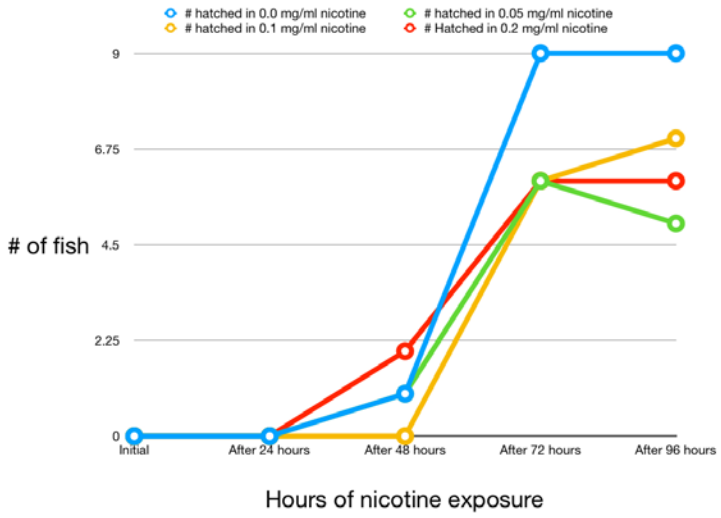

Fig. 2

Figure 2: the rate of zebrafish hatching after nicotine exposure; the results are similar to those of Fig. 1. It was not thought of when measuring these results, but this graph is actually a combination of hatched and alive. Any dead fish that had hatched were not a part of the data.

When graphed, results clearly show a trend in which zebrafish that were exposed to greater amounts of nicotine not only had more casualties, but also hatched quicker than the control. Those in the control group not only lost just a single embryo, but hatched slightly slower and started swimming first of the four groups, while those that were exposed to 0.2 mg/mL of nicotine lost

four and hatched too quick, as seen in the curvature of their spine (see fig. 4, bottom right) and and very little movement besides some slight fidgeting of their fins.

Although the results were expected, a chi-square analysis was done to prove the significance of the experiments results. The null hypothesis was a nice and rational 6.75. As the experiment called for four dilutions, and the mortality was recorded (fish can only be alive or dead, not both or neither), the degree of freedom was 3. With these numbers, the chi square value calculated out to a mere 3.99, or a 27.5 percent chance of the results being related to chance, and therefore insignificant. For results with a degree of freedom of 3 to be significant, the chi square value would need to be greater than the critical value of 7.82. That means the null hypothesis of 6.75 was accepted. This makes complete logical sense, due to some things that will be discussed in the discussion portion of this study.

Personal Observations:

Time of fertilization:

The zebrafish embryos are small, roundish balls with something comparable to an egg yolk in the center. The membrane is almost invisible, and no signs of anything remotely fish-like are noticeable.

18 hours after fertilization:

Ten of the zebrafish died within these first hours. Interestingly enough, the zebrafish in the smallest dilution of nicotine had the most “casualties” and the largest dilution had the second least. As predicted, the control had the very least, with only one found dead. The surviving embryos are now starting to develop, and share physical traits akin to those of a mosquito curled around a yellow ball.

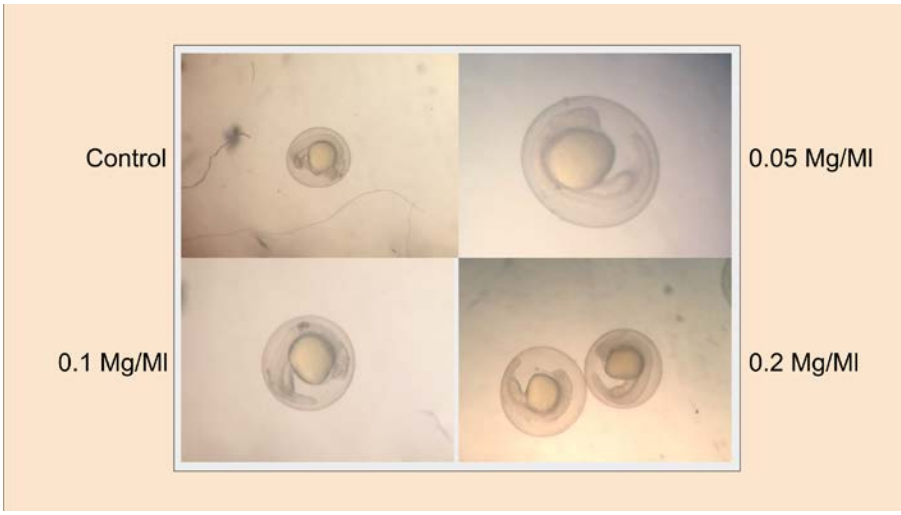

Fig. 3: the stage of zebrafish development after eighteen hours

48 hours after fertilization:

A few eggs have hatched now, and are looking good. Only one hatched in both the control and the 0.05 mg/mL dilution, but two hatched in the largest dilution. However, although there were more hatched in the largest concentration, they seemed frail and small, and had extreme curvature to their back. On the other hand, in the control and smallest dilution the ones that hatched are large and fairly straight. There is a clear difference between the two dilutions. The ones that had not hatched yet were now responding to some movement of the water in the wells and moving around inside the egg.

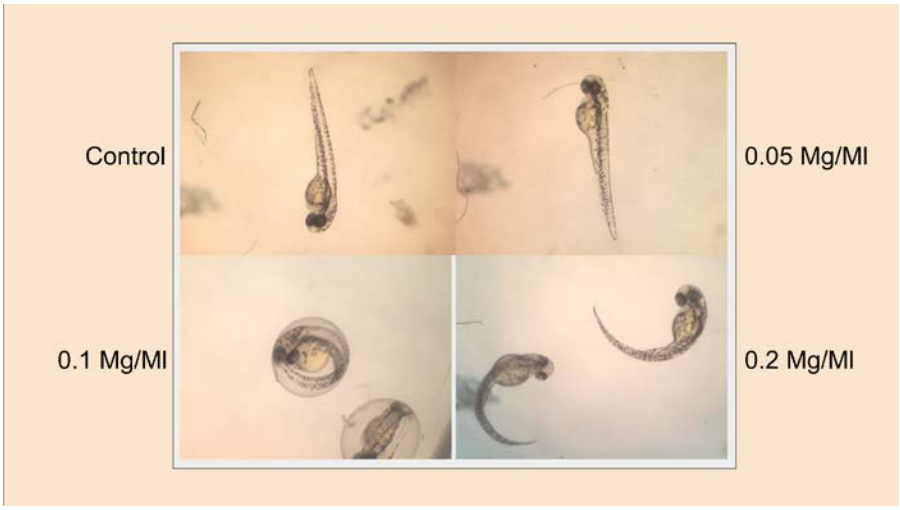

Fig. 4: the stage of zebrafish development after forty eight hours

72 hours often fertilization:

Most of the fish are now hatched and swimming around. However, some that have hatched have some degree of curvature. There are several with an almost 180° sideways curve, and one with a 80° upwards curve. others have a slight degree of curvature. The number of fish in each solution follows: none in control, one in the 0.05 mg/mL solution, one in the 0.1 mg/mL solution, and five in the 0.2 mg/mL solution. Although all seem healthy, two eggs may have broke open in the 0.05 mg/mL solution due to a transferring mistake, causing them to “pre-maturely hatch.” Now movement is common among the fish.

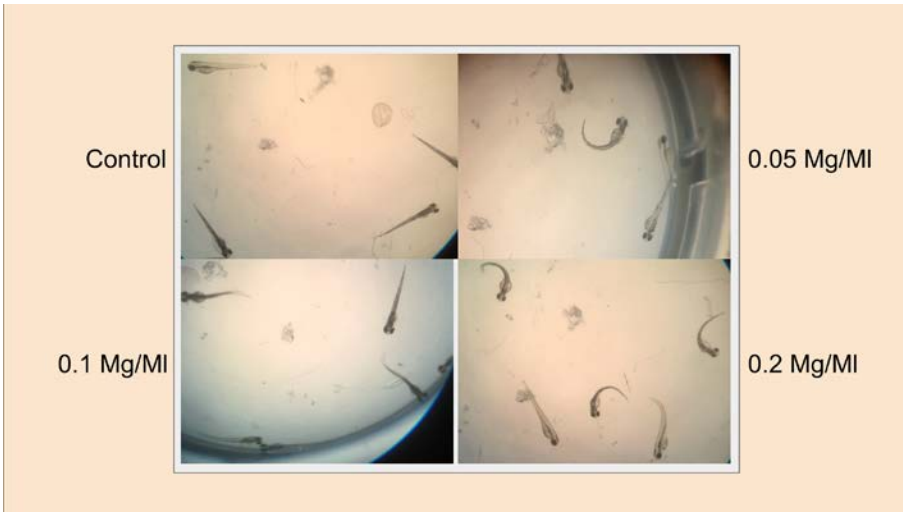

Fig. 5: the stage of zebrafish development after seventy two hours

96 hours after fertilization:

As the final day of the experiment, this will be the final observation. Immediately as the dishes were opened, a major difference was discernible. First, the 0.05 mg/mL and 0.2 mg/mL had two dead fish, one in each. It was also noted that they were not as responsive as the ones in the 0.0 mg/mL and 0.1 mg/mL solutions. Upon further inspection, the fish in the two different groups (responsive and unresponsive) had different heartbeats. Those that were almost catatonic had small, faint, and quick heartbeats that were almost indistinguishable from the actual fish, while those that zipped around the dish clearly had stronger, slower heart beats. Yet the main difference

between these two groups was in the solution itself. After the previous observations, it was noted while searching for similarities that there was a blue tinge to the water, showing a high concentration of methylene blue, the fungicide that was protecting the zebrafish. The head of the fish in those two solutions also had a deep blue coloring to them.

Discussion-

Nicotine is a stimulant, something similar to a drug that can lead to multiple health problems. It is extremely addictive and repeated exposure can lead to heart defects. Many people rely on this drug to remain calm, and become emotionally distressed when going through withdrawal. Because of this addictivity, women who smoke will, if heavy smokers, continue to smoke even if they become pregnant and if the nicotine found in cigarettes enters the developing fetus, it too may develop more severe heart defects, such as faster heart rate and a greater risk of strokes. The fetus also be subjected to premature birth or become a case of Sudden Infant Death Syndrome, or SIDS. According to this research, the zebrafish embryos should have had greater casualties in the higher concentrations and few casualties in the lower concentrations.

However, that was not observed. Instead, almost all of the concentrations of nicotine had around six zebrafish left alive, with the title of “most deadly” belonging to the lowest concentration? This seemed to be false, and that something other than nicotine was impacting the development of the zebrafish embryos. As this was an immediately confusing issue, observation was done immediately. Observations through the dissecting microscope showed a slight blueness on the foreheads of the baby fish, other than that confirming the same confusing results. Only when looking at it with the naked eye showed clear difference; the two concentrations with the fewest survivors, 0.05 mg/mL and 0.2 mg/mL, had a noticeably bluer tinge to the water, which must have given the blue coloring to the heads of the babies. It was also noted that few of the fish in the two blue wells actually responded to stimuli, although a heartbeat was seen. Given that methylene blue was a major chemical in the solutions, it was nearly certain that a larger concentration of the fungicide was used in those two solutions.With a second glance, it was also clear that there were physical differences as well. As shown in figures 4 and 5, the zebrafish in the two solutions had a

slight curve to their back and were incredibly inactive compared to their less blue counterparts, which swam all around their wells.

This is just speculation, but it is believed that if methylene blue is poisonous to zebrafish if exposed to high concentrations of it. This new hypothesis stems from those prior observations. If those zebrafish exposed to the higher concentration of methylene blue had a survival rate of 55% (0.05 and 0.2 mg/mL), as compared to the 80% (0.0 and 0.1 mg/mL) of those that had a lesser concentration, then, along with the obvious physical deformities, they must have been affected by said methylene blue. If that is the case, that would explain why the results were not significant. There also may have been some casualties caused by mistakes made while transferring eggs or the contaminated liquid. Two eggs broke open when the fine bore pipette almost sucked them up, and they were both in the 0.05 mg/mL solution. Therefore, it cannot be deduced whether or not it was nicotine, methylene blue, or mistakes that caused the results of this experiment. It may have also been because it was the first time anyone tried this, so mistakes made this time may not be made a second time.

Because of all this, it was impossible to decide on a single conclusive factor in the death of the zebrafish. Sadly, this was the caused by some major sources of error in the experiment. With the the rush to get each step done due to time constraints, many mistakes were made, and the effects of nicotine on development are still questioned by the authors. The experiment couldn't answer the original query's. One such mistake was breaking of two eggs, causing them to prematurely hatch. Another example would be the aforementioned methylene blue, which was found in different concentrations within each solution. Many of these complications wouldn't have happened if there weren't limitations, like the fact that there was only one trial of each solution, which made the experience with chemical experiments all more important as few mistakes could be made; however, that experience wasn't there, so there weren't any mistakes to learn from. To improve this experiment, those who conduct it should have an understanding of how to run the experiment, as well as know the steps and tips on how to isolate the testing to a single variable. They should also have multiple trials being tested for each chemical solution, as an average of say, three or four, is better than one.

In conclusion, this experiment was completely inconclusive on the effects of nicotine on the development of an embryo. Too many side factors played into the experiment and could not be deduced which factor caused what. Errors were made along the way, and some unavoidable issues came to light as the experiment went on. This was, however, an educational experience on the importance of precision and continuity when conducting an experiment.

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