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Developmental Deformities and Deaths In Developing Zebrafish Resulting From Nicotine, Vape Fluid Without Nicotine, and Vape Fluid With Nicotine

ABSTRACT

Zebrafish are utilized extensively in science due to their similar genome to humans. By testing zebrafish embryos, we can see how human embryos would potentially react and develop. However, little research has been done on the effects of vaping on either embryos type. This paper explores what deformities can develop as a result of both of these agents, along with pure nicotine compared to controlled groups. This paper also reports the deaths of embryos throughout the experiment as a result of the chemical agents. The zebrafish were kept in freshly diluted concentrations of each of the three chemical agents: nicotine, vape fluid with nicotine, and vape fluid without nicotine, for ninety-six hours post-fertilization for observation. Our research is of relevance because more and more teens and young adults are using electronic smoking devices. The irreversible effects that these devices are having on the body are yet to be known, however, new research is being done to determine the adverse effects. Thus the importance of our results, which showed death amongst the zebrafish in the vape fluid with nicotine, heart sac edema, slow reaction time, and a decreased heartbeat. Our work is different than that of others because we used three comparable chemicals that are prominent in the lives of many young adults due to the rise in underage consumption and studied the effects of each fluid to differentiate the severity of each product.

INTRODUCTION

We are conducting this experiment to test the effects of different chemicals on zebrafish embryos as it relates to humans. Vape fluid with nicotine, vape fluid without nicotine, and pure nicotine are chemicals that are growing in use among teenagers and young adults. Scott Gottlieb, the Food and Drug Administration Commissioner, stated that e-cigarette use has reached a level "nothing short of an epidemic proportions of growth." New data was also released anticipating a 77 percent increase in e-cigarette usage among teeanagers as compared to 2017 ("The Youth E-Cigarette Epidemic" 2018).

Additionally, in previous research conducted by the University of California, nicotine was determined to cause negative health effects on human embryos; "nicotine can have adverse effects on the development of the foetus during pregnancy and may contribute to cardiovascular disease," (World Health Organization, 2016). Research from Medical News Today is congruent with the results from the University of California. They discovered, "a study on zebrafish larvae found that aerosols produced by e-liquid caused heart defects," (Newman, 2017).

We are choosing zebrafish embryos due to their genetic similarities to humans. In fact, they share 70% of their genes to human embryos (Why Use the Zebrafish in Research, 2014). Furthermore, they reproduce quickly, giving us many test subjects in a short period of time. Their clear color makes it easy to view any deformities they might develop. Zebrafish embryos mature rapidly, showing results in less than four days (Why Use the Zebrafish in Research, 2014).

The zebrafish are being placed in different concentrations of nicotine, vape fluid with nicotine, and vape fluid without nicotine for at least seventy-two hours. Each chemical will vary in lower to higher concentrations in order for us to observe how the Zebrafish react with these chemicals at varying levels of exposure. We are predicting that the embryos exposed to higher concentrations of chemicals will develop more deformities, experience abnormal development, and have higher mortality rates in comparison to the embryos in lower concentrations of chemicals. Each day we will observe the zebrafish under a dissecting microscope and a compound microscope. The dissecting microscope will allow us to get the count of live fish, and the compound microscope will give us a magnified view of the deformities our fish have due to their exposure in the toxic chemicals.

MATERIALS

Firstly, the zebrafish embryos were provided to our class through the University of Milwaukee's Wisconsin Inquiry Based Scientist-Teacher Education Program. Before we received the embryos, they were fertilized at 9am, and then delivered to us at 1pm. Upon delivery, the embryos were rinsed and placed in instant ocean/embryo media solution. The other materials the program provided for the experiment included a stock solution of nicotine that was diluted to 0.01, 0.05, 0.1, and 0.2 mg/mL using instant ocean/embryo solution. The same procedure was done for the vape fluid with nicotine and the vape fluid without nicotine at the concentrations of 0.05, 0.01, and 0.2 mg/mL. Moreover, the instant ocean/embryo media solution was not only utilized to dilute the vape fluid, but was also utilized as the solution for our control group. Three beakers varying in size were used in this experiment to hold dead embryos, clean pipettes, and dirty pipettes. Two types of disposable pipettes had been used throughout the experiment in the sizes of 1.5 mm and 1 mm. The 1.5 mm pipette was exploited to transfer embryos to depression slides for observation in comparison to the 1 mm pipette which was exploited to dispose of and replace the solutions as the tip was small enough to avoid sucking up the embryos. Depression slides with a cover slip helped us to view the embryos. A compound microscope was used for close up viewing of the depression slides. A dissecting microscope served for general observation of the twelve well plate. The twelve well plate was used to hold the embryos in their designated concentrations which were labeled with a sharpie. The twelve well plate was placed in an incubator when not being examined, set at 28.5°C, to make sure that the temperature was not a factor in the development of our embryos.

SAFETY PROCEDURES

Latex gloves and goggles provided to us by our teacher were used at all times to avoid contact with the chemicals and to reduce the chance of confounding variables. Compression slides were cleaned before a new concentration of embryos were observed. Materials were cleaned and disposed of properly at the end of each day. We tied back our hair at the start of each day.

METHODS

When we started the experiment, the zebrafish had been fertilized for twenty-four hours. Ten Zebrafish embryos were placed into approximately 1 mL their respective concentrations (either 0.01, 0.05, 0.1, 0.2 mg/mL of nicotine, 0.05, 0.1, 0.2 mg/mL of vape fluid with nicotine or vape fluid without nicotine, or instant ocean/embryo media solution) using a clean pipette for each well. The embryos were viewed under a dissecting microscope and observations were recorded for twenty-four hours post fertilization. At forty-eight hours post fertilization, the first procedure completed was carefully changing out the solutions with a 1 mm pipette to avoid sucking up any embryos and replacing the fluids with fresh concentrations. Following the replacement, counts were taken under a dissecting microscope of hatched and dead embryos and recorded in a data table. All dead fish were removed from the wells and placed into the appointed beaker. Next, three fish per well were removed with the 1.5 mm pipette and transferred to a depression slide to be viewed under a compound microscope. Photographs of the fish were taken by placing a camera up to the lense of the microscope, which allowed for comparisons of the zebrafish exposed to environmental agents to the control groups. In addition to observing deformities, the heartbeats of the zebrafish were observed as well. A stopwatch was set for one minute and the heartbeats of the fish were counted for the duration of the time. Observations and heartbeats were recorded for forty-eight hours post fertilization. Seventy-two and ninety-six hours post fertilization, the third and fourth day of the experiment, the same procedure was followed regarding changing solutions and observing embryos. After observing the Zebrafish at ninety-six hours post fertilization, the zebrafish were euthanized using a bleach solution and disposed of accordingly. The procedures were done as stated to ensure the fish were being influenced by the correct chemical and there was no cross contamination. The experiment was carried out for four days in order to verify that embryos were truly being affected by the chemicals they were exposed to.

RESULTS

Even in day to day life, we are seeing the rise of teen vaping within our own school, and the research is congruent with this across the nation (Lavito, 2019). We tested the effects of different concentrations of nicotine, vape fluid without nicotine, and vape fluid with nicotine on zebrafish embryos and compared results to two control groups of zebrafish. What we found

corresponded with our hypothesis. Over the course of four days, more zebrafish in higher concentrations developed deformities or died compared to the control groups.

The zebrafish in instant ocean/embryo media solution developed normally. They all grew to have straight spines and tails, normal sized heads and eyes, and regular hearts (figure 1). At seventy-two hours post fertilization, we recorded an average heart rate of 120 beats a minute and fast reaction times across the board. They were swimming around in their wells, whereas none of the fish exposed to chemicals were moving.

The fish exposed to nicotine experienced a slower heart rate, 80 beats per minute, while the control group was around 120 beats per minute. We also observed enlarged brain stems and mild heart sac edema. The fish in nicotine concentrations developed drastic spinal curvature on top of their other malformations (figure 2). Deformities worsened as concentration increased.

The vape fluid with nicotine resulted in the most deaths among the zebrafish embryos. At ninety-six hours post fertilization, there were none alive in any of the concentrations. At seventy-two hours post fertilization the hatched fish showed enlarged eyes and extreme heart sac edema. Yet, the most obvious deformity among the embryos in the vape fluid with nicotine was multiple bends in their spines (figure 3). It was difficult to view other potential issues at ninety-six hours post fertilization because the hatched fish were swarmed by protists, making the image appear blurry (figure 3).

The zebrafish embryos in different concentrations of vape fluid without nicotine showed the most extreme deformities. Although their eyes were substantially larger than the control group, their heart and internal organs were underdeveloped. In addition, they showed severely yellowed brains and spines. However, their spines developed regularly in terms of shape, and stayed mostly straight (figure 4). At seventy-two hours post fertilization, the fish in vape fluid without nicotine had an average heart rate of seventy-two beats per minute and had almost no reaction time. When we poked them with a 1 mm pipette, roused them to swim, and transferred them to depression slides, they showed no signs of movement despite the heartbeat.

Overall, we found that nicotine, vape fluid without nicotine, and vape fluid with nicotine all negatively impact the development of zebrafish embryos. With such close relation to human embryos, we can assume that these chemicals would also negatively affect human babies in vivo. From the results on the zebrafish, we can predict similar deformities in humans, such as underdeveloped internal organs and bodies and craniofacial deformities.

Two chi square tests of independence were performed in order to examine the relationship between the exposure and death of fish. The first chi test of independence ran examined the relationship between the exposure of nicotine in comparison to the control and the number of dead fish. The two tailed P value equaled .1423 and was not statistically significant. We had to accept the null hypothesis even though our results showed that compared to the control the nicotine was the cause of death. One reason our data may not have been statistically significant was because of the small sample sizes. The second chi test of independence ran examined the relationship between the exposure of vape fluid with nicotine in comparison to the

vape fluid without nicotine. In this case the vape fluid without nicotine served as our control. The two tailed P value equal .0001 and was extremely statistically significant. This statistical test shows that the vape fluid may have had more effects on the zebrafish in comparison to the nicotine. Looking at the graphs and the table, this data is supported. In the graphs, at ninety-six hours post fertilization, the progression of vape fluid with nicotine and without nicotine, have fewer zebrafish alive than in the graph showing the progression of zebrafish in nicotine (figure 1). In the table, the percentages of zebrafish alive at ninety-six hours post fertilization is higher for nicotine than it is for vape fluid with and without nicotine.

The graphs and the table below are also an example of how our data supports the claim that the zebrafish in higher concentrations will experience more deaths over the course of four days than those in lower concentrations. As seen in Figure 1, there are more deaths at ninety-six hours post fertilization than at any other time frame. Of those deaths, most of them occur in the highest concentrations of the environmental agents. Outside of the control groups, every environmental agent experienced the death of a zebrafish. As the concentration increased, the number of deaths increased as well. Thus supporting our hypothesis.

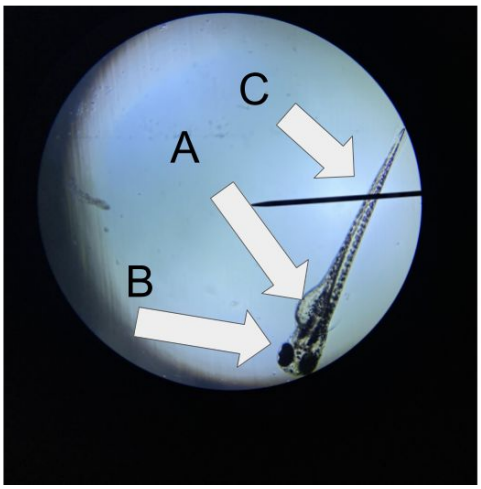

Figure 1
Image of a zebrafish in ocean/embryo media solution at 96 hours post fertilization. This zebrafish served as our control. Point A shows how the sac of a zebrafish should look. Point B demonstrates normal eyes, and point C shows a straight spine.

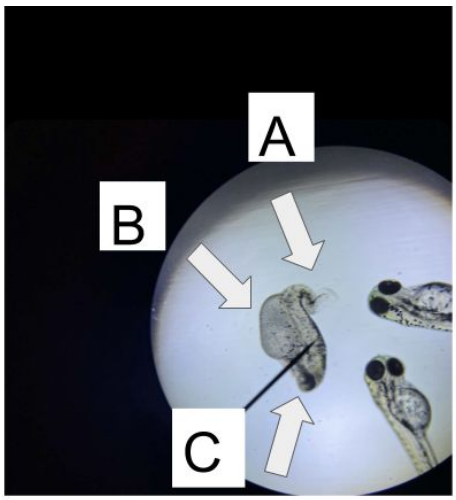

Figure 2
Image taken of three Zebrafish in a 0.2 mg/ml Nicotine concentration at 96 hours post fertilization.
Point A shows abnormal spine curvature, point B shows heart sac edema, and C shows that the eyes were not developed.

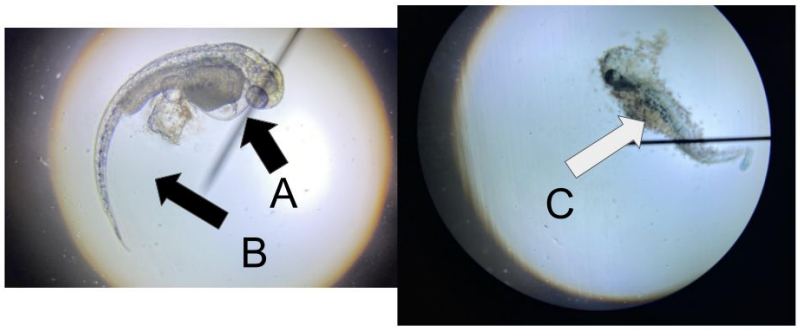

Figure 3
Image of a zebrafish in 0.2 mg/ml vape fluid containing nicotine at 72 hours post fertilization (on the left) and an image of a zebrafish in 0.2 mg/ml vape fluid containing nicotine at 96 hours post fertilization (on the right). Both Zebrafish are dead. Point A shows heart sac edema, and point B shows spine curvature. The zebrafish on the right is surrounded by protis (point C).

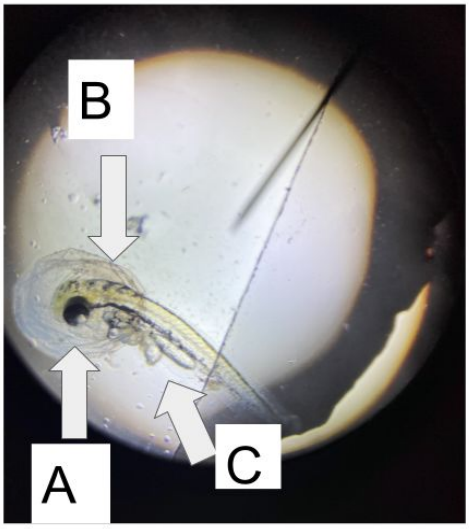

Figure 4
Image taken of a zebrafish in 0.2 mg/ml in vape fluid not containing nicotine at 72 hours post fertilization. Point A displays enlarged eyes, point B shows the yellow brain, and point C portrays the underdeveloped organs.

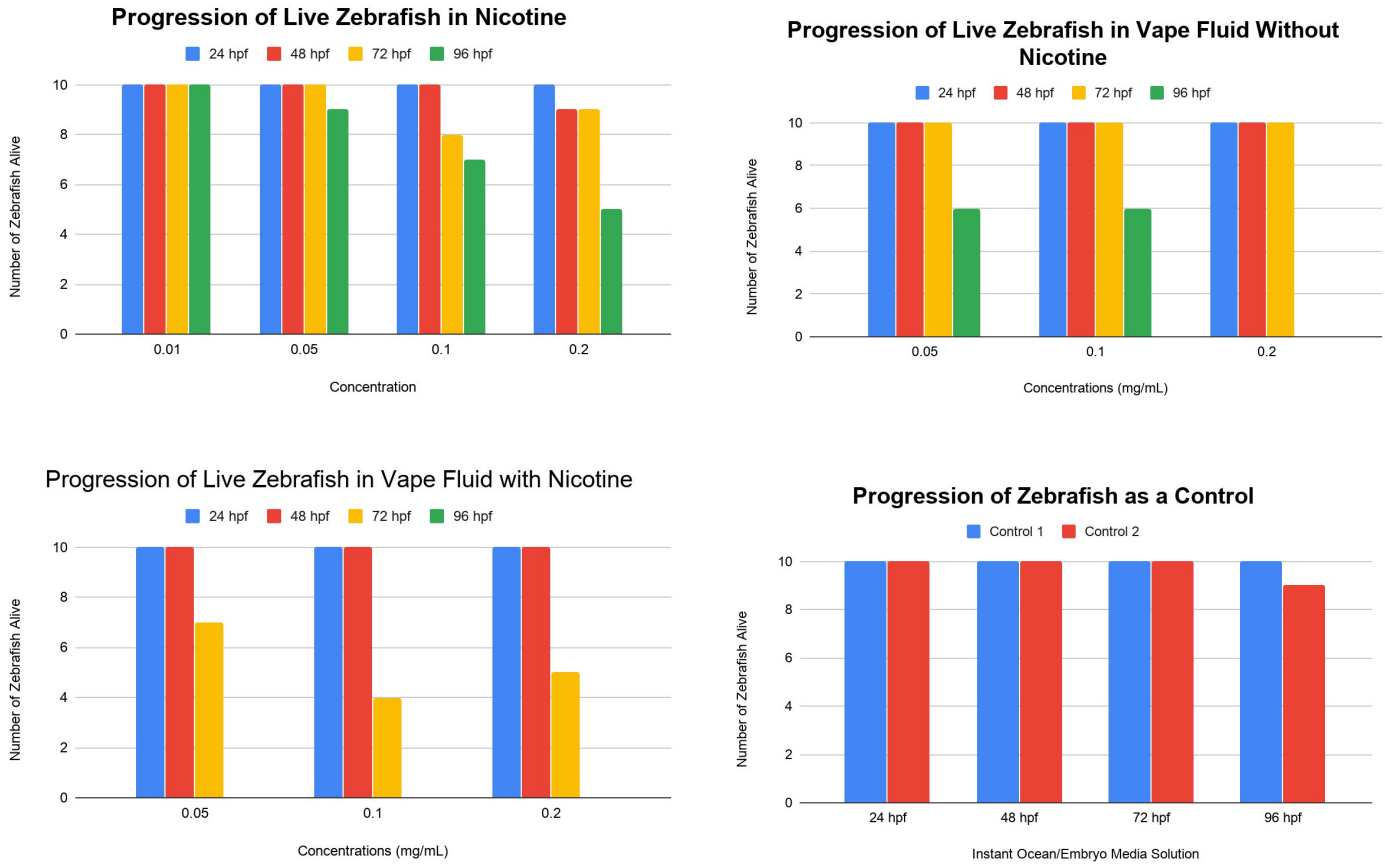

Figure 1 (Graph)

The graphs in this figure disclose the number of zebrafish alive in different concentrations at different time frames during the experiment. There is a graph for each treatment to allow comparison for the effects on the zebrafish. In the Progression of Live Zebrafish in Nicotine, there are very few deaths in the first seventy-two hours hours post fertilization and even at ninety-six hours post fertilization the deaths are still mild in comparison to the graphs representing zebrafish in vape fluid with and without nicotine. However, at ninety-six hours post fertilization the graph titled Progression of Live Zebrafish in Vape Fluid Without Nicotine shows fewer deaths than the graph titled Progression of Live Zebrafish in Vape Fluid With Nicotine. In comparison to the control, which out of the 20 zebrafish only one died, the other three graphs experience more deaths. It can be concluded that the exposure to the toxins, nicotine and vape fluid, is dangerous. Additionally, the data above supports our hypothesis in that it shows that in higher concentrations over a longer period of time there are higher mortality rates.

Treatment	Hours Post Fertilization	% Alive
Nicotine	**96**	**77.5 %**
Vape w/o Nicotine	**96**	**40%**
Vape w/ Nicotine	**96**	**0%**
Control 1	**96**	**100%**
Control 2	**96**	**90%**

Figure 2 (Table)

Figure 2 shows the percentage of fish alive for each treatment at 96 hours post fertilization. This table serves to show which treatment had the largest effect on zebrafish death. In addition, the amount of zebrafish alive at 96 hours post fertilization helped us to figure out values for the Chi Indepence test. For example, of the 40 fish in a nicotine concentration, at the end of the experiment 77.5 percent were alive.

DISCUSSION

The results that we found were congruent with our hypothesis. Zebrafish exposed to the concentrations of environmental agents developed abnormalities. A trend we discovered was that as the concentrations increased, more abnormalities occurred. The most significant result was found in the vape fluid with nicotine. The zebrafish exposed to these concentrations were all dead by ninety-six hours post fertilization, in addition to being swarmed by protists. They displayed enlarged eyes and extreme heart sac edema. Whereas these zebrafish in the vape fluid with nicotine had died off the most extreme deformities were found in the zebrafish exposed to vape fluid without nicotine. Their eyes were substantially larger and their heart and internal organs were extremely underdeveloped. They also displayed yellowed hued brains and spines. On top of these deformities, they had a slow reaction time to correlate with a slow heart rate. They were assumed dead at seventy-two hours post fertilization, but when placed under the compound microscope, they were proven to be alive by their very slow heart beat.

Multiple human errors were made throughout the process of this experiment that could have compromised the results. At least one embryo was killed in transfer from the well to the depression slide on our first attempt at viewing. When observing the zebrafish under the dissecting microscope, we could have miscounted or failed to remove a dead embryo or fish. Another error that was made was that not enough methylene blue was in the solutions, which caused the protists to develop and swarm the zebrafish. Moreover, as mentioned in the results,

the small sampling sizes may have also played a factor into why some of our results were not statistically significant. As a result of these errors, our live to dead ratios of the zebrafish may have been affected by unforeseen factors.

Our results confirm what was discussed in the introduction. All the embryos that were exposed to chemicals developed deformities. Of those deformities, fish that matured in higher concentrations had malformations that were much more severe. All three of the chemicals that were used are growing in use among teenagers and young adults. Nicotine and vape fluids come in a variety of flavors that teenagers are using in E-Cigarette devices. As previously mentioned in the introduction, a study by the University of California found that human fetuses experienced adverse developmental effects when exposed to nicotine throughout pregnancy (World Health Organization, 2016). For this reason, there needs to be more work done to find what the effects of these chemicals are on the human body, so that the people can be educated and aware of what they are consuming.

Further studies need to be conducted to find what the effects of nicotine, vape fluid with and without nicotine have on the human body. As stated in the introduction, zebrafish and humans share a similar genome, however it is not a complete match. This means that although abnormalities can be seen on developing zebrafish embryos, researchers cannot be certain of what the chemicals may do to the body of a human teenager. The American Lung Association states that human lungs mature between the ages of 20 and 25 (Lung Capacity and Aging, 2019). But what does this mean for teenagers who inhale nicotine and vape fluid? A study performed on a larger scale could be beneficial in terms of research compared to the small amount of zebrafish we used.

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