How Nicotine Affects a Developing Fetus Using Zebrafish Embryos

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Abstract

The information given is to show what really happens to a developing fetus when it is exposed to nicotine. There are many harmful risks when a pregnant woman smokes a cigarette or e-cigarette. This analysis is an attempt to resemble the effects of nicotine on a developing fetus. The zebrafish embryos are subjected because it closely resembles a developing human fetus. The data will show closely the consequence of smoking while pregnant. Five zebrafish embryos in four different wells were exposed to three different concentrations of nicotine. Everyday they were examined and observed. The end results demonstrated the effects nicotine has on embryonic development. With the survival and hatch rates demonstrated, it was found that nicotine is indeed harmful to a developing human embryo. It was not only determined that nicotine is catastrophic to a human embryo, but the grown, developed human body too. The increased dark spots on the zebrafish embryos indicate traces of soot, similar to a human that smokes. The seizures an embryo was experiencing demonstrated the neurological effect of smoking during pregnancy on a human developing fetus, such as addiction and including seizures.

Introduction

In the US, one in every six deaths are a result of smoking. Every year those deaths sum up to approximately half a million Americans (Illinois Institute for Addiction Recovery, 2003). Nicotine, found in many tobacco products, is not directly cancer causing, though it is very harmful and very addictive. Nicotine reaches the brain within eight to twenty seconds after smoking (Felman, 2018). Low doses of nicotine can act as a central nervous system stimulant. Higher doses cause an increase in nerves, heart rate, and the blood vessels to contract (Illinois Institute for Addiction Recovery, 2003). Nicotine can be absorbed many of ways; smoked, sniffed, and chewed. Within 2 hours of use, half of the nicotine remains in the body's systems. Some of the properties in nicotine including pharmacokinetic properties enhance the level of its abuse (Felman, 2018). Soon after the exposure of nicotine, there’s a kick caused in a part of the adrenal glands resulting in discharge of epinephrine, also known as adrenaline (Psychology Today, 2019). From this research it can be drawn that once an individual becomes a regular user of nicotine and intern addicted, it can be difficult for them to quit.When women that are addicted to nicotine become pregnant, it is often hard for them to stop using it so quickly, resulting in many problems for a developing fetus. The smoke and chemicals from a cigarette can damage soft tissues of an unborn baby’s brain and lungs. When the baby is born with prior exposure to nicotine it can have various and serious development issues (Georgia Department of Public Health). The baby may suffer from low birth weight, high blood pressure, type two diabetes, behavioral issues, brain development issues, cleft palate, or cleft lip (Felman, 2018). Other issues that can occur due to smoking while pregnant include, but are not limited to preterm birth, still birth, and miscarriage. To further assess the effects of nicotine on a developing fetus, an experiment will take place in which zebrafish embryos will be exposed to different concentrations of nicotine during development. Zebrafish embryos are being used as they have similar embryonic developments and similar biological traits to that of developing human fetus. They are also transparent and develop outside of the mother's body, making them easily accessed (University of Oregon, 2013). For the experiment, it is hypothesized that if zebrafish embryos are exposed to different concentrations of nicotine, then the zebrafish embryos exposed to the

highest concentration of nicotine will experience more deformities, premature births, and have a lower survival rate compared to embryos exposed to lower concentrations of nicotine.

Materials and Methods

Materials

- Stock solutions of nicotine (3 concentrations: 0.05mg/mL, 0.1mg/mL, and 0.2mg/mL)
- Beaker for dead embryos and liquid disposal (1)
- Sharpie (1)
- Instant Ocean/Embryo Media Solution (1 bottle)
- Transfer pipette (2-3 skinny and wide)
- 3x4 multi-well plate (1)
- 28.5°C incubator (1)
- Glass slide (1)
- Compound Light Microscope (1)
- Stereoscope (1)
- Chemical tape (2 four inch long pieces)
- 20 Zebrafish
- Pencil & data sheet
- Camera for pictures

Methods

On day one of the zebrafish experiment, a clean and sterilized 3x4 well plate must be obtained. Place two pieces of tape across the lid of the well plate so that the bottom two rows of the well plate are covered. On the tape covering the middle row of wells, label the second, third, and fourth wells with the different concentrations of nicotine, 0.05mg/mL, 0.1mg/mL, and 0.2mg/mL respectively. The first well on the upper most left side is the control and should be filled with 1 mL of Instant Ocean Solution using a wide pipette. The experimental wells (to the right of the control) should be filled with 1 mL of the corresponding labeled nicotine solution concentrations, starting at the second well and working across to the right would be 0.05mg/mL, 0.1mg/mL, and 0.2mg/mL. Using a narrow pipette, place approximately 5 embryos into each well containing either the control or nicotine solutions. Record the exact amount of living embryos on a data collecting document. Take note that none will be hatched on day one. Observe each well of embryos under a microscope, record all observations. Place the well plate in an incubator overnight, set at 28.5°C.

On days two through four, remove the well plate containing the embryos from the incubator. Remove dead embryos from the plate with the narrow pipette, be careful not to removing living embryos (the dead embryos will no longer move and may appear fuzzy and white). Deposit the dead embryos into the waste beakers. Count the remaining embryos and any fish that have hatched and record on the data collecting document. Change the well solutions by removing the solutions from the wells (it may help to tilt the plate so that the embryos settle to the bottom, then the liquid at the top is easier to remove). After the old liquid has been removed, replace the solutions with the original concentrations. Fresh solution is provided daily so that the waste from the embryos is removed and the embryos have fresh oxygen. Make sure to always use a new,

clean pipette for each solution so there is no cross contamination of the solution concentrations. Next, place the plate under the microscope and record any qualitative and quantitative data, and make sure to take pictures. Once all observations and pictures are taken, return the well plate containing the embryos to the incubator.

On day five, remove the well plate containing the embryos from the incubator. Remove dead embryos from the plate with the narrow pipette, be careful not to removing living embryos (the dead embryos will no longer move and may appear fuzzy and white). Deposit the dead embryos into the waste beakers. Count the remaining embryos and any fish that have hatched and record on the data collecting document. Be sure to not to leave any information out; record all data, qualitative and quantitative. Be observant of any movement, deformities, and significant behavior. Once all observations and data is recorded, place all embryos and fish in a waste container and dispose of all organisms in an ethical manner.

Safety Precautions

When handling chemicals be sure to wear gloves. When changing embryos solution carefully tilt the well plate so no embryos get into the waste being removed. Always use clean pipettes when handling solutions and zebrafish (do not cross contaminate).

Results

The purpose of this research project was to evaluate the effects that different concentrations of nicotine have on a developing fetus. It was hypothesized that if zebrafish embryos are exposed to different concentrations of nicotine, then the zebrafish embryos exposed to the highest concentration of nicotine will experience more deformities, premature births, and have a lower survival rate compared to embryos exposed to lower concentrations of nicotine. The independent variable was the different concentrations of nicotine going into each well. This change affected the dependant variables of the zebrafish embryos survival rate, hatch rate, and deformity. The control within the experiment was the well containing instant ocean/embryo media solution (a solution with 0.00 mg/mL of nicotine).

The different concentrations of nicotine showed different results and effects on the embryos. The embryos in the control well had very faint black spots scattered all over. All of the embryos from the higher concentrated wells had darker more defined spots. The higher the concentration was, the darker and bigger the spots and eyes were. The highest concentration showed a texture such as dark soot near its stomach and lungs. The lowest concentration of nicotine after the last day showed the hatched fish seizing out consistently. The most common effect on the embryos from nicotine exposure was getting dark black spots all throughout their body. The higher the concentration of nicotine, the more spots there were; the spots didn't get darker, they simply became more noticeable and appeared to be more solid. The eyes of the zebrafish embryos also became a great deal larger and darker as the concentration of nicotine increased.

On day three, in the highest concentration there was one alive and three dead. The control had three alive and zero dead consistently to day four. In the end, the highest concentration had no survivors. The hatched versus unhatched was very significant. Three of the embryos in the

highest concentration died before they could even be hatched. The one that was left on the last day was hatched, but not alive. The control embryos had the earliest hatchings. Comparing the death rate of the 0.1 mg/mL (medium concentration), it had 2 survivors and a slower death rate than the higher and lower concentration. Overall, the control had the most hatched and alive in the end (3 hatched and alive). There were none left unhatched.

In the well with the lowest concentration (0.05 mg/mL) the one embryo alive on day five experienced seizures after being exposed to nicotine. The zebrafish embryo of the medium concentration (0.1 mg/mL) became shriveled, small, and unproportional. Well 3 (0.1 mg/mL), had a high rate of deaths before the embryos hatched. Such data relates to how if a pregnant woman smokes, she can have a miscarriage. Finally, well 4 (0.2 mg/mL) demonstrates the worst effects of nicotine on the developing embryo. Most of the embryos died before they had the chance to hatch (stillborn). Only one zebrafish survived through the growing stage to hatch, but died soon after (shown in figure 1 and 2 as light purple).

The data displayed closely justifies the purpose for the experiment, to show the physical effects of nicotine on an embryo. It also relates back to our initial hypothesis, which was that the higher concentrations would cause more problems.

Figure 1:

Figure 1 Graph on the number of zebrafish alive after being left in different nicotine concentrations over a period of 96 hours.

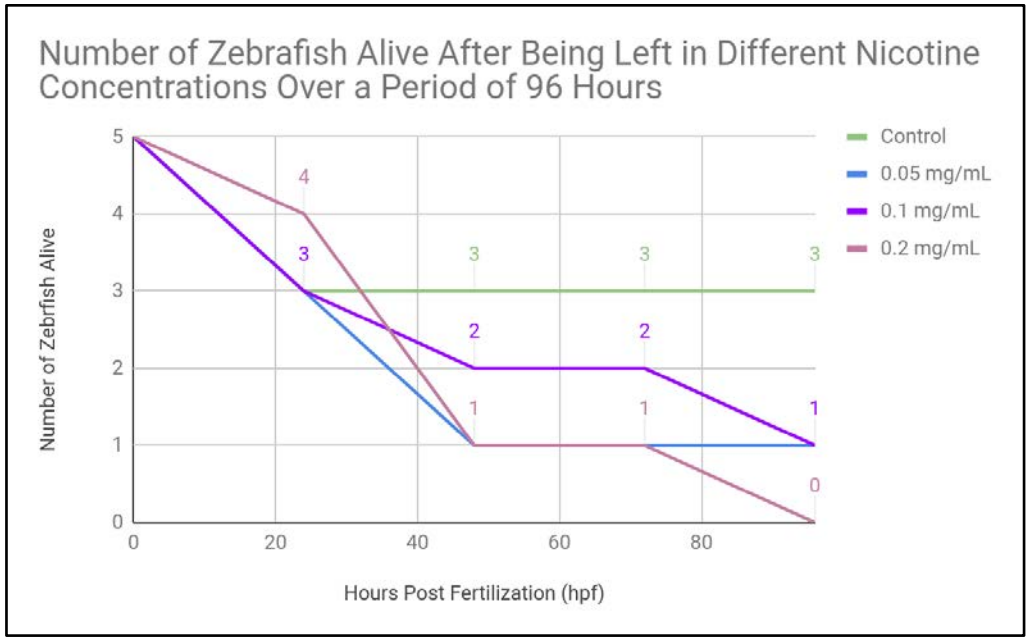

Figure 2:

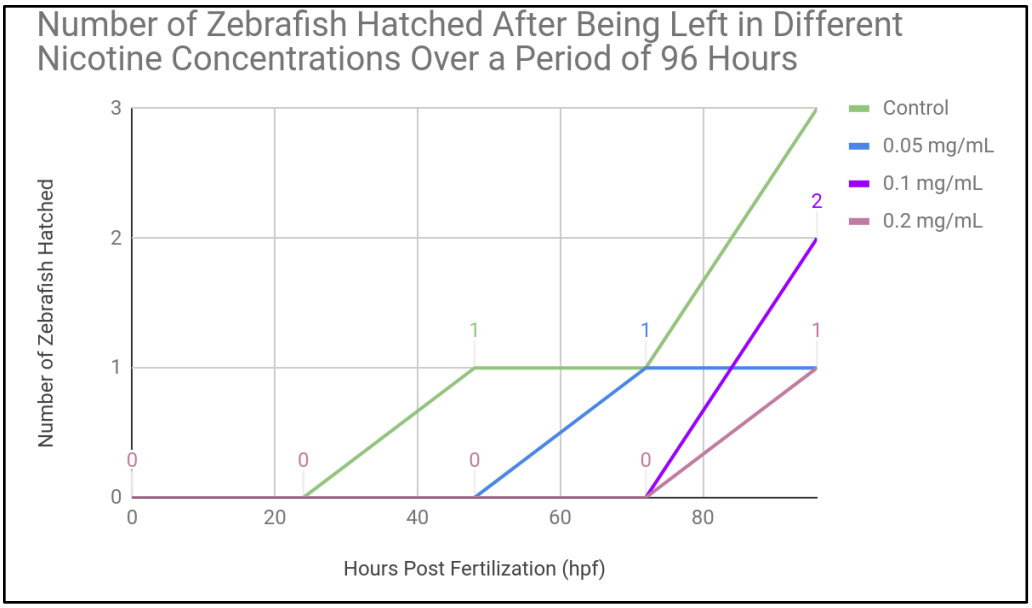

Figure 2 Graph on the number of zebrafish hatched after being left in different nicotine concentrations over a period of 96 hours.

Table 1:

Number of Embryos **Alive vs Dead (Alive/Dead)** at Different Concentrations of Nicotine over One Week

Time	hpf	Control Group (well #1) Alive / Dead	Experimental Group Concentration 0.05mg/mL (well #2) Alive / Dead	Experimental Group Concentration 0.1mg/mL (well #3) Alive / Dead	Experimental Group Concentration 0.2mg/mL (well #4) Alive / Dead
Day 1	0 hpf	5/0	5/0	5/0	5/0
Day 2	24 hpf	3/2	3/2	3/2	4/1
Day 3	48 hpf	3/0	1/2	2/1	1/3
Day 4	72 hpf	3/0	1/0	2/0	1/0
Day 5	96 hpf	3/0	1/0	2/0	0/1

Table 2:

Number of Embryos **Hatched vs Unhatched (H / U)** at Different Concentrations of Nicotine over One Week

Time	hpf	Control Group (well #1) H / U	Experimental Group Concentration 0.05mg/mL (well #2) H / U	Experimental Group Concentration 0.1mg/mL (well #3) H / U	Experimental Group Concentration 0.2mg/mL (well #4) H / U
Day 1	0 hpf	0 / 10	0 / 10	0/10	0/10
Day 2	24 hpf	0/3	0/3	0/3	0/4
Day 3	48 hpf	1/2	0/1	0/2	0/1
Day 4	72 hpf	1/2	1/0	2/0	1/0
Day 5	96 hpf	3/0	1/0	2/0	1/0

Figure 3:

Control and all well plates

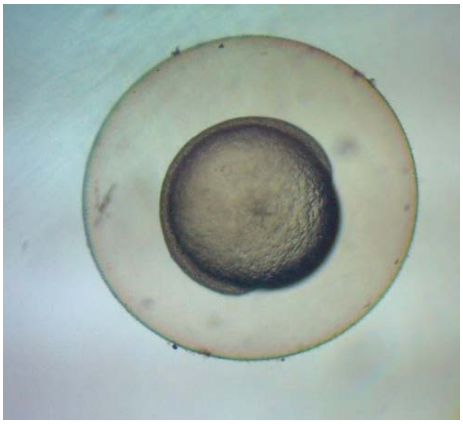

Figure 3: Zebrafish embryo on day 1 (same for each well plate)

Figure 4:

Concentration of 0.05 mg/mL

Figure 4: **Well plate 2**, lowest concentration of nicotine (0.05 mg/mL)

Picture 1 (top right): Day 2, zebrafish is unhatched and alive

Picture 2 (top left): Day 4, zebrafish is hatched and alive

Picture 3 (bottom): Day 5. Zebrafish is hatched and alive, shell visible

Figure 5:
Concentration of 0.1 mg/mL

Figure 5: **Well plate 3**, medium concentration of nicotine mg/mL)

Picture 1 (left): Day **2,** two zebrafish embryos unhatched and alive
Picture 2 (right): Day 3, zebrafish embryo is unhatched but alive, with a shrunken shell

Figure 6:
Concentration of 0.2 mg/mL

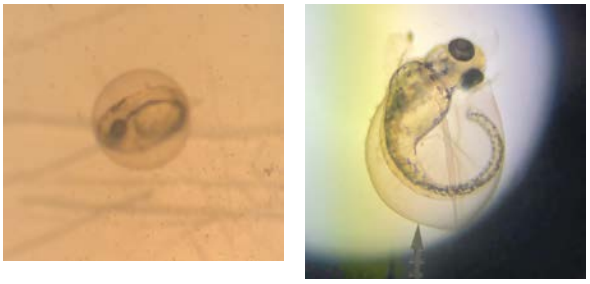

Figure 6: **Well plate 4**, highest concentration of nicotine (0.2 (0.1 mg/mL

Picture 1 (left): Day 2, zebrafish embryo is unhatched and alive
Picture 2 (right): Day 4, embryo is hatched and alive

Discussion

The results reflect the initial hypothesis which was that if zebrafish embryos were exposed to different concentrations of nicotine, then the zebrafish embryos exposed to the highest concentration of nicotine would experience more deformities, premature births, and have a lower survival rate compared to embryos exposed to lower concentrations of nicotine. The data supports the hypothesis because there were no embryos alive in the highest concentration of nicotine compared to the control with three alive. This data shows significance because it reflects the effects of nicotine on pregnant women who smoke. With the many embryos that died before hatching, this first justifies miscarriages, stillborn births, and other birth complications. The concentrations of nicotine in a juul are superior to the concentrations that were used in the experiment. The concentrations illustrate the number of cigarettes smoked, the amount of nicotine into the body differs depending on the amount of cigarettes or packs smoked per week. The experiment can show that even the lowest amount of nicotine can cause issues. The embryo in the low concentration well was having seizures. Going back to day five, the one embryo left in well 4 (0.2 mg/mL concentration) was halfway out of the yolk sac and the embryo was not alive. This evidence reflects the stillborn birth that can occur when a pregnant woman smokes consistently. In addition, the baby may also suffer from low birth weight, high blood pressure, type two diabetes, behavioral issues, brain development issues, cleft palate, or cleft lip (Felman, 2018). Although the experiment didn't allow for the specific developing issues to be detected, they may have been apparent as the zebrafish matured. It is still to be concluded that there were obvious differences from the control compared to the embryos exposed to nicotine.

The hypothesis stated that the highest concentration of nicotine would result in the most premature births and deformities, but it really caused the most deaths to the embryos. The lowest concentration caused the embryos to seize continuously. This could be the possibility of brain issues and a form of addiction to nicotine. The medium concentration was the one that caused

less deaths than both concentrations. The effects were not as noticeable except for dark spots near the stomach and lung area. Furthermore, the highest concentration showed the most deformities. The one surviving, hatched embryo appeared curled up and not as mobile as the less concentrated wells. This zebrafish embryo also had a very small fin curled back. The curled back fin mirrors the risk of deformities to the human fetus, as a result of the smoking pregnant woman.

The results relate back to the original experiment and show that with the use of nicotine, a fetus or embryo could suffer from birth defects, seizures or neurological defects, physical deformities, and premature births or death in the womb. Each concentration caused different effects that got more serious the more days that went by. Although, the experiment came to its limitations. As the outside and physical effects could be recorded, the outcome of behavioral consequences was hindered.

Overall, the zebrafish exposed to the different nicotine concentrations were not as healthy as the zebrafish with no exposure. As stated in the hypothesis, it was expected that the embryos in the higher concentrations would have a lower survival rate. This was proved when the control (unexposed) had the most alive on day five (three embryos alive) and the highest concentration had none alive on day five.

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