Continued Exposure to Nicotine and its Effect on the Development of Zebrafish Embryos

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Abstract
15-20% of pregnant women inhale the drug nicotine through cigarettes. Due to this, nicotine has been known to cause problems in embryonic development. To test the effects of nicotine, zebrafish embryos were placed in varied concentrations of nicotine. The embryos were observed everyday till euthanization, euthanization happened at the end of the fourth day. Some results included: disfigurement, underdevelopment, enlarged heart, and discoloration. These results provided further evidence supporting the hypothesis that nicotine affects babies and infants.

Introduction
Zebrafish have seven periods of embryogenesis: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and the hatching period. All of these stages are an important part of a developing zebrafish embryo as the end result will dictate how it will live its life and whether or not it reproduces. During all of these stages the embryo goes through various physical changes. During the beginning three stages the embryo begins by cleaving, after cleaving the cytoplasm moves towards the animal pole, segregating the blastocysts. During the end of the third stage the ectoderm begins migrating to the outside. In the fourth and fifth stage the germ ring is formed which dictates where bodily organs will be formed, then the dermis, vertebrae and skeletal muscles are formed, the beginning of the primary organs also develop along with the neural cord and the notochord, the body also moves for the first time. During the last two stages the body axis begins to straighten, the nervous system is hollow and is expanding anteriorly, the circulatory system develops and the heart beats for the first time, blood also starts circulating throughout an enclosed system. At the end of the last stage the zebrafish will begin to hatch out of their embryo and begin to mature to a fully healthy zebrafish adult. Substances such as drugs, alcohol, and even pollution can affect any of these stages and cause the zebrafish to not live to its expected development. This experiment will look at the effects of nicotine on the embryonic developmental stages.

Humans can be as equally affected by nicotine in the same ways other animals such as zebrafish are, which is why it's important to know more about the drugs that are affecting the younger generation. Nicotine, or otherwise known as nicorette or nicotrol is a toxic colorless or yellowish oily liquid that is the main active substance of tobacco. It acts as a stimulant in small doses but can block the action of autonomic nerve and skeletal muscle cells in larger doses. Smoking during pregnagcy has also shown to be linked to premature births, motor, sensory, and cognitive deficiencies in toddlers and infants, findings have also shown that maternal smoking may cause in external behavioral problems in older infants according to R Wickström *Effects of Nicotine During Pregnancy: Human and Experimental Evidence.*

The purpose of this experiment was to study and demonstrate the effects of nicotine in the development zebrafish embryos and how these effects correlate to the effects of nicotine in a human fetus. This experiment is extremely relevant in today's society, according to the article; *Effects of Nicotine During Pregnancy: Human and Experimental Evidence* 15 to 25% of women smoke during pregnancy [20,94,98]. Recently the effects of smoking during pregnancy has become more known, this causes women to turn to alternative sources such as smokeless

tobacco. 90% of women in India consume smokeless tobacco according to: *Effects of Nicotine During Pregnancy: Human and Experimental Evidence*. This experiment will help inform women the effects nicotine can have on their children's development.

It was hypothesized that embryos exposed to high concentrations of nicotine would suffer from slowed down development and underdevelopment. The experiment concluded that the fish affected by the nicotine were underdeveloped and suffered many abnormalities such as spinal deformities and discoloration.

Materials and methods

Materials

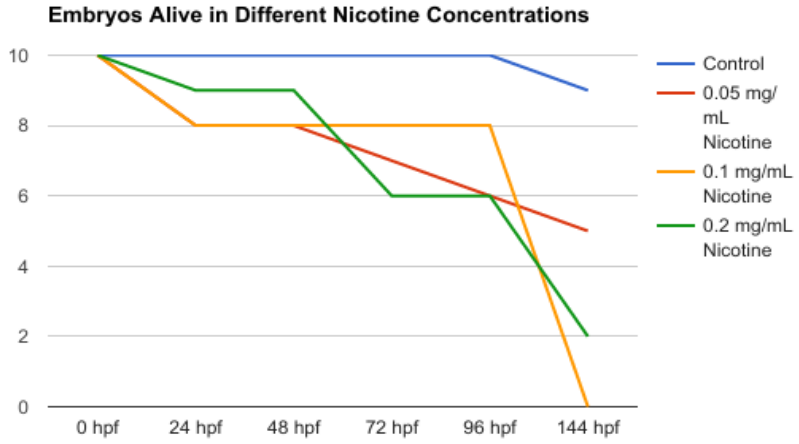
- One permanent marker
- One plate with wells
- One mL pipette
- One minimum bore 1.5mm pipette for moving embryos around
- Depression slides
- One compound microscope
- One dissecting microscope
- One bottle of Instant Ocean/Embryo Media
- One bottle of 0.05 mg/mL Nicotine solution
- One bottle of 0.1 mg/mL Nicotine solution
- One bottle of 0.2 mg/mL Nicotine solution
- One beaker for waste
- One incubator set at 28.5 degrees celsius
- Zebrafish embryos

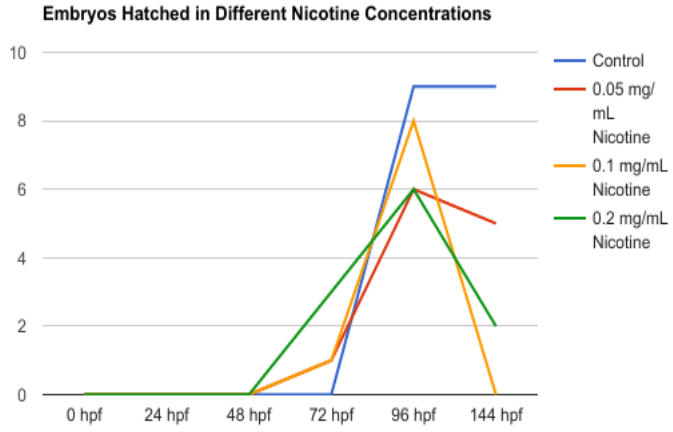
Methods

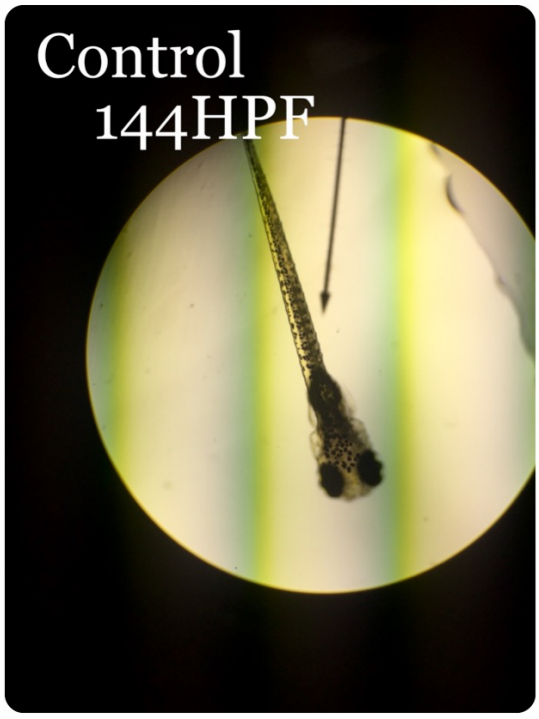
The well plate cover was labeled as to always know what well contained what, all four wells were filled with 1mL of Instant Ocean with the wide bore pipette. The first well only contained embryo media in order to act as our control, the second well was filled with 1mL of the 0.05mg/mL nicotine solution, the third well with 1mL of 0.1mg/mL, and the fourth well with 1mL of 0.2mg/mL nicotine solution. Ten embryos were transferred to each well using the minimum bore pippette. Dead embryos were disposed of in the waste beaker. The number of embryos contained in each well were precisely double checked using the dissection microscope.The embryos were returned to their correct respective well in the plate,the plate itself was placed in a 28.5 degrees celcius incubator. On days two and three the well plate was removed from the incubator in order for the embryos to be observed. Dead embryos along with waste solution was discarded into the waste beaker. The wells were then replaced with their respective measurements of the nicotine solution. The remaining embryos along with the newly hatched fish were taken count of using the dissection microscope. Individual embryos and hatched fish were placed on deppresion slides and viewed under a compound microscope. All observations along with quantitative data was recorded in the data sheets. All embryos were retired to their respective wells and the well plate was returned to the incubator which was set at 28.5 degree Celsius. On day four the plate was removed from the incubator for observations. Surviving

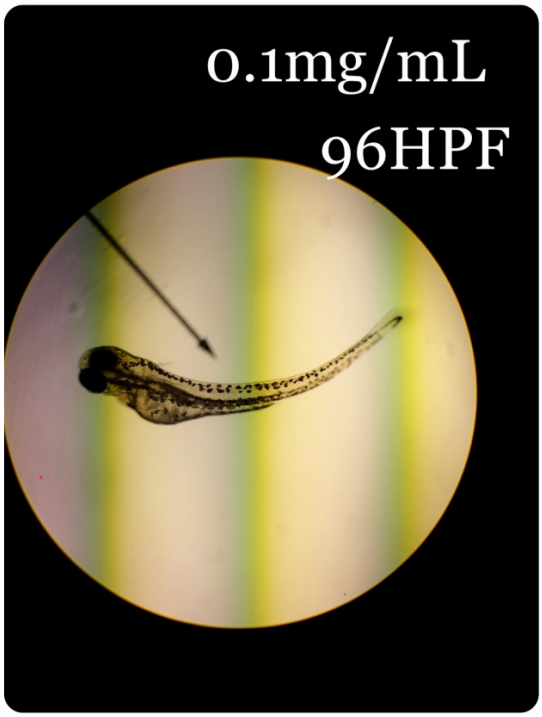
embryos and hatched fish were counted using the dissection microscope. Individual embryos and hatched fish were placed on dimensions slides and observed under the compound microscope. All observations were recorded in the data sheets. All of the embryos, hatched fish, and solution waste were removed from their wells and placed in the waste beaker. All of the embryos and hatched fish were euthanized at the end of day four.

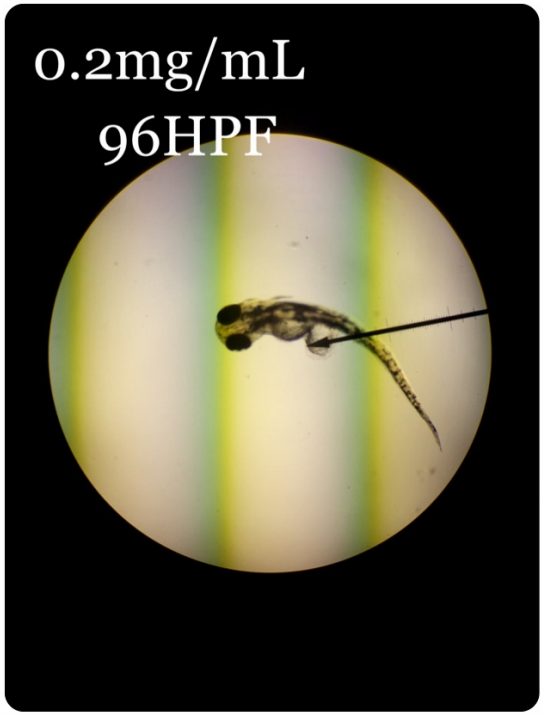
Results

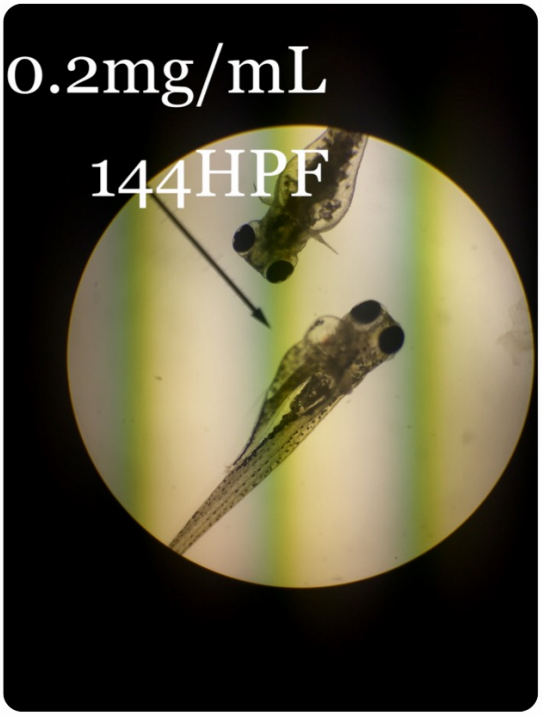





Control
144HPF


0.1mg/mL
96HPF


0.2mg/mL
96HPF


0.2mg/mL
144HPF

The experiment above was conducted in order to study the effects of nicotine in developing children. It was hypothesized that embryos exposed to high concentrations of nicotine would suffer from slowed down development and underdevelopment. For the experiment, the embryos were separated into their respective wells, the control with the embryo media and the rest of the wells with successively denser concentrations of the nicotine solution, the independent variable being the concentration of nicotine and the dependent variable being the number of live and hatched embryos along with their appearance and behavior.

The embryos residing in the nicotine solutions hatched a day earlier than the control and the expected hatching timeframe for zebrafish. The fish residing in the nicotine solution started hatching at 72 hours post fertilization. The fish affected by the nicotine developed sooner but had more deformations (mainly in the tail and backbone) and differences in color and patterns compared to their healthy counterparts. The nicotine affected fish clearly stopped developing color wise after 48(hpf). The most clear deformations were the tails, obviously deformed 96(hpf) in the 0.1mg/mL solution and even more so in the 0.2mg/mL solution 96(hpf). Compared to the control, the fish in 0.2mg/mL had quickened heartbeat at 72(hpf). Towards the end of the experiment at 92(hpf) the fish in the 0.2mg/mL solution had slower heartbeats that also beat irregularly. At 144(hpf) the 0.2mg/mL batch had the most problems, some of the fish had facial deformities, lopsided spinal disfigurements, were underdeveloped, had large heart sacs, smalls fins, large eyes, and they also had the least color out of the rest of the fish who resided in different solutions.

The results supported the hypothesis and provided further knowledge on the effects of nicotine during embryonic developmental stages. Most of the data we needed was found in the results but information on cognition, mental disabilities, societal issues, and behavioral issues was not found as it is not feasible with the current technology. There was a small problem encountered on day 4 (at 72 hpf), due to the careless mistake of a distracted researcher four newly hatched fish in 0.2mg/mL, one fish in 0.1mg/mL, and one fish in 0.05mg/mL were placed in the waste beaker by accident when the fish along with the solution waste were sucked up by the small bore 1.5mm pipette. Further precaution was taken the rest of the experiment and waste solution in the pipette was double checked before being placed in the waste beaker.

Discussion

The experiment resulted in underdeveloped fish,obvious physical disfigurements, and premature hatching. Underdevelopment and physical disfigurements could be related to its rapid and abnormal development when compared to the control, the body itself may be growing at a furious pace leaving the physical structure struggling to catch up any way it can hence the disfigurements. Rapid development may also lead to premature hatching which is also evident in many babies exposed to nicotine. These results provide even more solid evidence against nicotine and its effects.

These results support research previously conducted on nicotine and its effects. This experiment concluded that embryos exposed to consecutively denser concentrations of nicotine were born prematurely, suffered from discoloration, and had various physical deformities. These

problems became more frequent and severe in higher concentrations of nicotine, further proving the bad effects of nicotine in developing embryos.

This experiment provided data on the physical effects of nicotine but further research should be done on the mental and societal impacts of nicotine in infants and children. Further research and other experiments should be done on the issue of the discoloration of fish as it could provide further insight of the many effects of nicotine.

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