The Effect of Ethanol on Zebrafish Embryos

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Abstract

Ethanol, also known as grain alcohol or ethyl alcohol, is defined as a "colorless alcohol made from grain and sugar that is volatile...and burns easily, and is used in medicines and other substances and in alcoholic drinks" (Cambridge University, 2017). In numerous experiments, the effects of alcohol on zebrafish is extremely similar to the effects of alcohol on human fetuses, due to similar vascular systems (Matsui, 2006, October). Studies have been done showing the effects of Ethanol on adults. Ethanol is a chemical that when consumed by humans at high doses, has been shown to induce complications. According to National Institute on Alcohol Abuse and Alcoholism (2001), "Alcohol depresses nerves that control involuntary actions such as breathing and the gag reflex (which prevents choking). A fatal dose of alcohol will eventually stop these function." An assumption can be made that those with a high blood alcohol concentration will have involuntary movements due to numbed nerves. On the other hand, one can only imagine the effects of ethanol on the developing nerves of human embryos. Studying how alcohol affects zebrafish embryos can help to provide a better understanding of the effects alcohol has on human fetuses. Therefore, the purpose of this experiment is to discover the effects of ethanol on the zebrafish embryos. Through background information, prior to the experiment, it was discovered that zebrafish embryos have numerous deformities when exposed to ethanol.(Green, 2016) In turn, all vertebrate fetuses including human will have little growth and the possibility of death when exposed to high concentrations of alcohol during embryonic development. This was studied by submerging ten zebrafish embryos into solutions containing various amounts of ethanol (0mM, 30mM, 100mM, 300mM), for a total of forty experimental zebrafish embryos. The embryos were then observed once every 24 hours over the course of five days. Observations were recorded about the size, shape, movement, and development stages of the embryos. The embryos in the control well appeared to have more development when compared to those in the higher amounts of solution used. In 300mM, most of the embryos had died 72 hours post fertilization. Through further experimentation the hypothesis for the experiment can be solidified.

Introduction

In 2010, the U.S spent 249 billion dollars due to excessive alcohol use of citizens. Of that money, 5.5 billion dollars was spent on problems related to alcohol consumption during pregnancy. According to the National Library of Medicine (2015), "Drinking during pregnancy can cause brain damage, leading to a range of developmental, cognitive, and behavioral problems, which can appear at any time during childhood. Fetal Alcohol Spectrum Disorders (FASD) is the umbrella term for the different diagnoses...." A breakthrough was made on facial indicators of children exposed to ethanol during development in 2003. However, these characteristics are often only visible after the baby is born. Doctors are still in search for indicators of Fetal Alcohol Spectrum Disorders during embryonic development, in hopes of giving earlier diagnosis (Jacobson, 2003). The question was then raised of the characteristics of

an embryo exposed to ethanol during the embryonic development, and for this reason, the experiment was based on different concentrations of ethanol on zebrafish embryos. This would then reveal the effects of ethanol on the survival rate and the developmental rate of the embryos. In the prospect of finding distinct characteristics or indicators of an embryo exposed to ethanol during embryonic development. Humans and zebrafish are both vertebrae. Ergo, zebrafish and humans will share similar biological traits, such as their developmental process, genes, and certain behaviors (Badman, 1995). Due to these similarities, the way that ethanol affects zebrafish embryos will affect human embryos in a comparable way. Unfortunately due to the many complications that alcohol can have on developing human fetuses, a hypothesis was formed. If the concentration of alcohol increased, then the survival rate and the development rate of the zebrafish embryos would decrease, because of the exposure to high amounts of alcohol causing extreme deformities.

Materials and Methods

Materials

- Stock solutions of Ethanol (30, 100, 300 mM Ethanol)
- Beaker for dead embryos and liquid disposal
- Sharpie
- Instant Ocean/Embryo Media Solution
- Disposable pipette, minimum bore, 1.5 mm
- Disposable pipette, 1mL pipette
- Plate with wells
- 28.5℃ Incubator
- Depression slide with slip cover
- Dissecting and compound microscope
- Latex-free gloves
- Safety Goggles

Methods

On day one of the zebrafish lab, the essentials for the experiment are required. The first day of the lab is mainly for set up. The first item needed is a clean 3x4 well plate is needed. Following, on a flat surface, label the four wells based on the concentration of ethanol in each well,0mM, 30mM, 100mM, and 300mM respectively. Then, retrieve required embryos from the oceanic solution. Using a 1 mL disposable pipette, add ten zebrafish embryos very carefully to each well. Be certain that the embryos are alive. Living embryos should be obtuse and transparent. It is common to miscount the embryos, so be careful. However, the use of a bright light can be held up to the well plate, making it easier to see the small embryos. Afterwards, to ensure that the results are accurate, the leftover solution is removed using a 1 mL pipette.

Recount the zebrafish embryos to ensure that there are still ten embryos in each well, improving the accuracy of the experiment. An ethanol concentration of 0mM, 30 mM, 100mM, and 300mM is needed next. Using a clean 1.5 mL disposable pipette, add 1 mL of concentration into the correctly labeled well. Proper safety techniques should be enforced handling chemicals such as ethanol. Safety goggles and latex free gloves are highly recommended for this experiment. Using a dissecting and compound microscope, initial observations should be made. Taking in account of initial color, size, and movement of the eggs. Findings should then be recorded and pictures should be taken of the zebrafish embryos. Following observations, the well plate needs to then be placed in an incubator at 28.5 degrees Celsius.

On day two, 24 hours post fertilization, observations and pictures of the embryos need to be taken. Using either a compound microscope or a dissecting microscope set on 25mm (highest setting). Observations should be taken on any deformities of the embryos size, shape, and color, as well as the number of embryos hatched, alive, and dead. Afterwards, the wells need to be clean and replaced with new solution. Remove all dead embryos and the old embryos of hatched zebrafish. A dead embryo can be identified by a black mass inside the embryo. Likewise a hatched zebrafish can be identified as dead if black marks are inside the fetus or the fetus appeared to be fuzzy. Dead zebrafish should not be identified by the characteristic of no movement. Newly hatched zebrafish will have little to no movement, however, they are still alive. Taking in account of these indicators, dead embryos should be removed using a 1 mL disposable pipette and a dissecting and compound microscope. While removing dead embryos, the wells should be cleaned from the lowest solution to the highest solution in prevention of cross contamination. The removed dead, and old solution should then be placed into a clean 100 mL beaker. It has been found difficult to remove all the dead embryos and the solution without accidentally removing a living embryo. As precautions, after cleaning each well, observe the beaker under the compound microscope to make sure all removed embryos, and hatched zebrafish are in fact dead. After carefully removing dead embryos, new solution is added to each well using a 1 mL pipette. The same concentration of solution was added to each well. When the procedure is finished, place the well plate into an incubator set at 28.5 degrees Celsius.

On day three of the lab, observations should be recorded using a dissecting and compound microscope set at 25mm (the highest setting). Determine the living and the embryos, based on color and the lack of movement. Most of the hatched zebrafish should have movement. Observations should be taken on the movement of the hatched zebrafish. In addition observe the color of the live zebrafish, the bodies of the hatched zebrafish should be clear. Afterwards, the old solution and dead zebrafish are to be removed from each well, using a 1 mL disposable, very small tipped pipette. The solution is to be removed from wells 0mM, 30mM, 100mM, and 300mM in that order, so as to not contaminate any of the wells. The old solution and dead zebrafish should be placed into a beaker. After each well is cleaned, examine the beaker of waste to ensure no living zebrafish are in it. If any living zebrafish are found, carefully place the embryo back into the proper well. Once the wells are cleaned, the same concentration of ethanol

solution needs to be added into each well. After each well has new solution, the well plate should be placed into an incubator set at 28.5 degrees Celsius.

On day four of the zebrafish lab, using the same dissecting and compound microscope as previous days make observations on the zebrafish size, shape, color, and the number of dead zebrafish. It is common for the zebrafish to have increased movement as opposed to the previous day. Look for any deformities and the stage of development the zebrafish are in. Heartbeats should be visible at this point in the experiment, due to the transparency of the embryos and zebrafish.

Once the embryos and zebrafish are determined to be dead or alive, the old solution and dead zebrafish are removed in order from the wells with the lowest concentration to those with the highest concentration of ethanol. The solution and zebrafish are removed using a 1 mL pipette with a small, precise tip. The old solution is moved into a beaker that also holds the dead fish. The beaker is closely examined to make sure no living zebrafish are accidentally placed into it. If any living zebrafish are in the beaker, they are moved back into the proper well. After the solution and dead zebrafish are removed, new solution with correct concentration of ethanol is placed into the same well. Once each well has new solution, the well plate is placed into an incubator set at 28.5 degrees Celsius.

On day five of the experiment, the observations need to be recorded using the dissecting and compound microscope. Placing well underneath the microscope, zebrafish should be examined. Majority of zebrafish that are still alive should be hatched. After making observations, determine the live and dead zebrafish. Using a 1 mL pipette with a precise tip remove dead zebrafish and old solution. Following the extraction of the dead zebrafish, they should be placed into a beaker. Always examine the beaker after the cleaning of each well to ensure there are no living zebrafish. If there are any zebrafish in the beaker, they are to be placed back into their appropriate well. After cleaning of the well plate is completed, refill the well plate with the correct amount of ethanol solution.

Results

This experiment was done to show how different amounts of ethanol affect the zebrafish embryos. In the experiment, there was a control well with 0mM of ethanol (instant ocean solution). The ethanol was distributed between three wells at different concentrations: 30mM, 100mM, and 300mM. Ethanol was used as the independent variable in this experiment and zebrafish mortality and developmental irregularities were the dependent variables. Setting up the experiment in this way created a better understanding of how different amounts of ethanol affect developing zebrafish embryos. Shown in figure 1, there was a higher amount of ethanol more embryos survived. These results lead to the reexamination of the early procedures of this experiment. It was determined that unforeseen variables may have altered the experiment and in turn, altered the results. Errors may have been something has miniscule as a contaminated pipette, or completely altering, such as addition of a higher concentration of ethanol to the wrong well. Although it is shown in figure 1 that as the concentration of ethanol increased the survival

rate increased, the development rate did not coincide with the survival rate. In figure 4, it can be seen that the zebrafish are still in their embryonic egg casings. On the contrary in figure 5, zebrafish in lower concentrations were already hatching. Showing the slower developmental rate of embryos exposed to higher amounts of ethanol.

Although the results of the experiment were skewed, based on the chi-square in figure 3, data collected on day five is significant. The null hypothesis for this chi-square is that the likelihood of survival of the embryos is the same for all treatments. This hypothesis was accepted on day 5 based on the degree of freedom being in range of validity. Based off the chi-square table, there is a 90% likelihood that if this experiment was repeated the same results would occur. Meaning that although the procedure was affected by unforeseen variables was corrected by day 5 of the experiment.

Figure 1: Zebrafish Experiment Observations

Treatment	Well #	# of starting fish	24 hours post fertilization		48 hours post fertilization		72 hours post fertilization		96 hours post fertilization	
Ethanol			#hatched	#live	#hatched	#live	#hatched	#live	#hatched	#live
Control	1	10	0	7	4	3	3	2	1	1
30mM	2	10	0	7	6	3	3	2	2	1
100mM	3	10	0	9	0	7	6	7	7	5
300mM	4	10	0	10	1	9	5	6	3	3

Figure 1 shows the data for each day of the experiment; the days, the different wells, the number hatched, and the number of living Zebrafish.

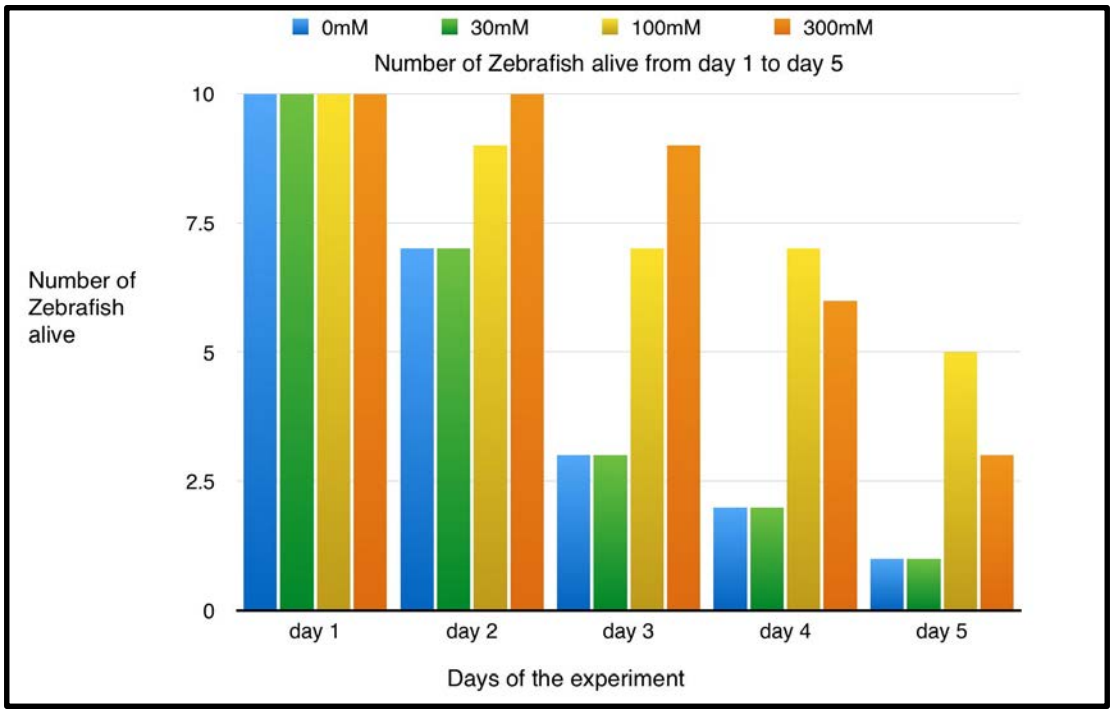

Figure 2: Shows the data received for each day of the experiment,; the different wells, the days, the number of living Zebrafish, and the number hatched.

Chi Square Day 5

Treatment	Live	Dead	Total for Rows
Control (0 mM)	1(.66)	0 (.33)	1
30 mM	1(.66)	0 (.33)	1
100 mM	5 (4.66)	2 (2.33)	7
300 mM	3 (4)	3 (2)	6
Total for columns	10	5	15

x1+ x2= .5714

Figure 3 is the Chi Square for day five of the experiment. The first column shows the treatment that was used. The second column shows the number of living zebrafish in the well. The number of living zebrafish includes zebrafish embryos and the zebrafish that have hatched. The third column shows the number of dead embryos in each well. The fourth column shows the total number alive in each well. The numbers in parentheses show the number of zebrafish expected to be either alive or dead.

Figure 4: Day 3, 300mM well

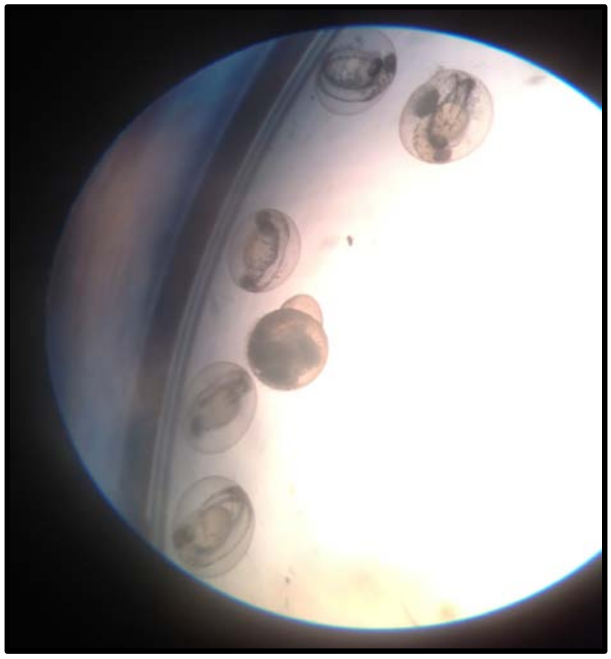

Figure 4 shows the zebrafish eggs that are in the 300mM well.

Figure 5: Day 4, 100mM well

Figure 5 shows one living zebrafish in the 100mM well on day four of the experiment.

Discussion

This experiment provided a deeper understanding of zebrafish embryo development. It also created a new perspective on how human fetuses develop under the impact of alcohol. As a result of research it was discovered that the vascular system in zebrafish embryos is similar to the

vascular system in human embryos as they are both vertebrates (Matsui, 2006, October). In light of this evidence a logical conclusion was made; the impact of ethanol on zebrafish embryos would be similar to the way that ethanol affects human embryos. Due to research done prior to the experiment showing that "Human fetuses which are exposed to ethanol during their development face many abnormalities such as fetal alcohol syndrome, failure to thrive, and other defects that affect their chance of survival" (Carvan III, 2004, September 11), a hypothesis was formed that as the concentration of ethanol increased the number of zebrafish embryos that survive would decrease. The data collected during experimentation failed to support this hypothesis. The data from the experiment showed that zebrafish embryos had a better survival rate in higher concentrations of ethanol, which contradicted the hypothesis of the experiment. Which lead to the conclusion that something had been contaminated in the experiment. Human fetuses that are exposed to ethanol during their development have a higher death rate. This means that the same fact should be true about zebrafish embryos because both are vertebrae. Leading to the conclusion that the data in this experiment was inaccurate. As a result, a reevaluation of this experiment was done. It appeared that the results of this experiment were backwards. In this data as the concentration of ethanol increased, the survival rate of the zebrafish increased. This outcome led to the conclusion that somewhere in the experiment, wells were cross contaminated. This conclusion is supported by an experiment done by DB labs. In DB labs experiment, they also studied the effects of ethanol on Zebrafish embryos. In DB labs the results showed that the zebrafish in the control well developed at an normal rate. On the other hand, our results were the complete opposite, proving that somewhere in this experiment wells were cross contaminated (Cebra, 2001, August 2). How they were contaminated is unknown, however, precautions can be made to ensure cross contamination does not happen in future experiments. It is highly recommended to use a clean pipette. If a dirty pipette is used you will cross contaminate the wells of the experiment. In addition, when changing the solution in the well plate, always go from the lowest concentration to the highest concentration to ensure that there is no cross contamination. Due to the errors in this experiment, the hypothesis that if the concentration of alcohol increases, then the survival rate of the zebrafish embryos will decrease, because of the over exposure to high amounts of alcohol, could not be proven. With further research, experimentation, and proper precautions, this hypothesis could be confirmed.

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