The Effects of Ethanol on Developing Zebrafish Embryos

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Abstract:

This experiment was performed to test connections between ethanol and the hatch rate of zebrafish which may be used to correlate to human embryonic development. In this experiment, there were four wells containing ten zebrafish embryos in each and were exposed to different concentrations of ethanol. These concentrations include 0 mM (Control variable), 30 mM, 100 mM, and 300 mM. Over the course of 96 hours, their development was monitored and recorded as the zebrafish hatched. Research consisted of observing zebrafish through a dissecting microscope and checking if the fish were living, hatched, or dead. That data was recorded and the zebrafish were put into incubation overnight. That data was later analyzed and compiled into tables and graphs. The hypothesis was that ethanol slows development of zebrafish. However, data that was observed during this experiment differed from what was hypothesized. It is believed that data obtained through the experiment had a few outliers that skewed the data. These outliers could have skewed the data away from disproving the null hypothesis of the Chi square analysis. Based on the Chi square data, results did not fully support the hypothesis it seems as if there is a connection between ethanol and slower embryonic development of zebrafish. If the null hypothesis was disproved, there would be enough evidence to prove that ethanol has a negative impact on embryonic development. This would also be true for human embryonic development because of similar genes between humans and zebrafish.

Introduction:

Zebrafish were tested on because they have similar genes to humans and react equally to certain substances. This means that zebrafish can be tested on instead of humans and a correlation between results can be drawn. According to Wellcome, "Sequencing of the entire genetic makeup of the zebrafish has revealed that 70 percent of protein-coding human genes are related to genes found in the zebrafish and that 84 percent of genes known to be associated with human disease have a zebrafish counterpart." This is why zebrafish are great organisms to use as a model organism to research diseases or substances. By using zebrafish in experimental tests, scientists can predict the results a particular disease or substance will have on a human, depending on how it affects zebrafish. High levels of ethanol are dangerous for humans, and can be lethal. "Alcohol depresses the central nervous system and causes low blood glucose (sugar). Children who drink alcohol can have seizures and coma; they could even die" (Soloway, R. (2011)). So, what can zebrafish embryonic development tell us about human development?

This study was performed so information could be gathered on how ethanol affects the developing body without actually testing it on people. For example, according to an article by the March of Dimes, "Brain damage and problems with growth and development", this information further warrants the need for further testing, for this study the substance ethanol (C_2H_6O) was used in different amounts on zebrafish to understand how ethanol affects embryonic development.

The experimental hypothesis was that if zebrafish were put into different amounts of ethanol, then zebrafish in the wells with a higher concentration of ethanol would develop slower than those in lesser amounts of ethanol. This would happen because alcohol has been scientifically proven to harm the growth of fetuses.

Materials and Methods:

The materials used consisted of a well plate which has sixteen wells (four of which were used), 40 zebrafish (divided into four wells, ten fish in each), 1 mL each day of four different solutions- 0 mM (the control solution) of ethanol, 30 mM, 100 mM, and 300 mM, pencil and paper to record results, two different types of pipettes, one with wider tube for zebrafish embryos, and one with smaller tube for just replacing solutions. 5 beakers, four were 100 mL, which were filled with different concentrated solutions, and one more which was 50 mL for waste, day old ethanol, dead fish, and gloves for a safety precaution. An incubator was used in between the times when the zebrafish were studied. There was a dissecting microscope used to look closely at the zebrafish and an iPad used to take close up photos through the microscope of the zebrafish as they developed. All of the materials were provided by the Wisconsin Inquiry based Scientist Teacher Education Partnership (WInSTEP) Program, which is part of the NIH Science Education Partnership Award (SEPA) Program administered by the University of Wisconsin–Milwaukee and the Children's Environmental Health Sciences Core Center.

Day 1 (after 0 hours)

First, a large tip pipette, used for collecting embryos, well plates, a microscope, and documents that were needed for research were collected. Gloves were put on for safety. Ten embryos were placed in each of the four wells. Residual liquid was removed and replaced with 1 mL of each unique solution in the four wells (0 mM in well one, 30 mM in well two, etc.). Caution was taken not to spill any fluids in the wrong well or on the floor, and triple-checked that there were 10 embryos in each. Embryos were observed using the dissecting microscope and videos and pictures were taken with an iPad to document movement and growth. Data on the rates of development of zebrafish was taken based on how many zebrafish were living, hatched, or dead through the dissecting microscope (the zebrafish were considered dead if there was a black dot inside of the zebrafish while the zebrafish were in their embryonic state or if the zebrafish were curled up into a shriveled up ball once hatched from the previous state). The embryos were put into the incubator (at 28.5 degrees Celsius).

Day 2 (after 24 hours).

The well plate was removed from the incubator. Then researchers replaced the solution in each of the wells. This was done by using a small tip pipette. Caution was taken as to not inadvertently remove any of the embryos. Dead embryos were removed using a large tip pipette, three dead zebrafish were removed that day. Pictures were also taken this day to document the growth of the zebrafish. Data on the rates of development of zebrafish was recorded based on how many zebrafish were alive, hatched, or dead through the dissecting microscope. The embryos were put back into the incubator (at 28.5 degrees celsius).

Day 3 (after 48 hours).

The well plate was removed from the incubator. Solutions were replaced in each of the wells. This was done by using a small tip pipette so it doesn't extract the embryos. Care was taken as to not inadvertently remove the embryos. No zebrafish died this day. Pictures were taken to document the growth of the zebrafish. The numbers of zebrafish that hatched were documented. Data on the rates of development of zebrafish was taken based on how many zebrafish were alive, hatched, or dead through the dissecting microscope. The embryos were placed back into the incubator (at 28.5 degrees Celsius).

Day 4 (after 72 hours).

The well plate was removed from the incubator. The solution was replaced in each of the wells. This was done by using a small tip pipette so it doesn't extract the embryos. Care was taken as to not to extract the embryos. No zebrafish died this day. Pictures were taken to document the growth of the zebrafish. The zebrafish were all hatched by 72 hours after the experiment started, and that information was recorded. Data on the rates of development of zebrafish was taken based on how many zebrafish were alive, hatched, or dead through the dissecting microscope. The embryos were put back into the incubator (at 28.5 degrees celsius).

Day 5 (after 96 hours).

The well plate was removed from the incubator. The solution was replaced in each of the wells. This was done by using a small tip pipette so it doesn't extract the embryos. Care was taken as to not to extract the embryos. One zebrafish died this day and this data was recorded. Data on the rates of development of zebrafish was taken based on how many zebrafish were alive, hatched, or dead through the dissecting microscope. The living zebrafish at the conclusion of experiment were moved into an aquarium. Because there were no changes in the zebrafish, photos were not taken that day. Later a Chi Square analysis was performed to test for independence between concentration of ethanol and rate of development.

Results:

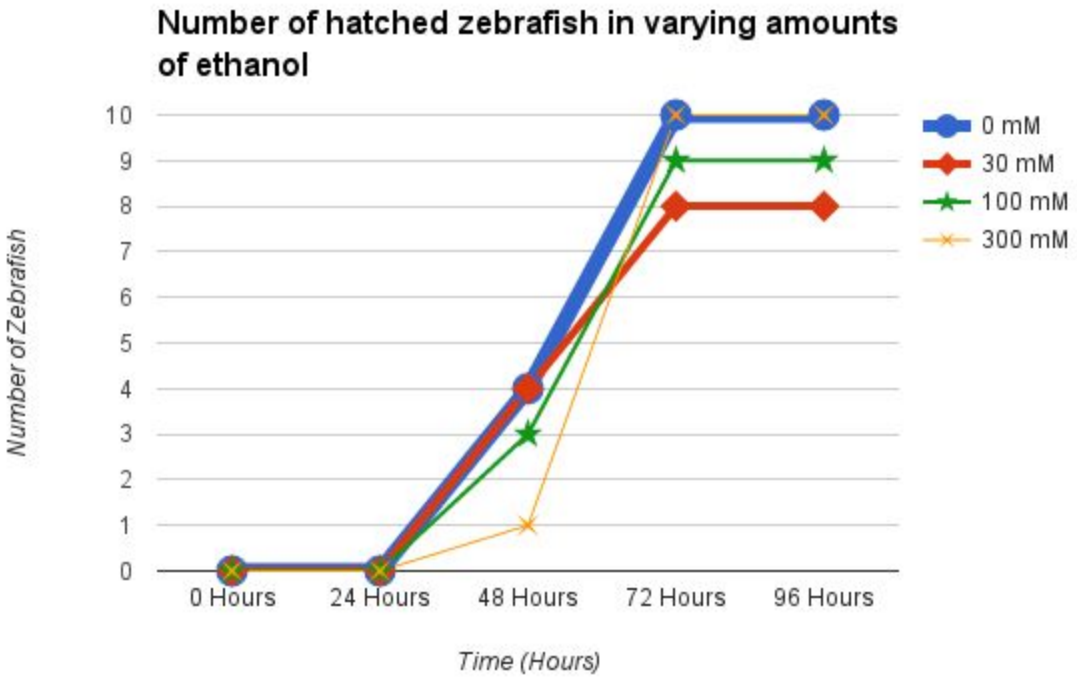
The experiment was based on finding out if ethanol had effects on growth/development of zebrafish embryos and if ethanol does have an effect on them, what is it? The embryos were set up in wells and everything possible was set as the same for all four wells except that the wells contained different concentrations of ethanol. This was done so that it was easily seen how many zebrafish lived, hatched, and died in each different solution. The independent variable was the amount of ethanol in the different wells and the dependent variable was the rate of development of the zebrafish. The control solution was the 0.0 mM solution, which contained no ethanol. The controlled variables of the experiment were, the size of the wells, amount of fluid in each well, amount of time in the solutions, and same millimolars of solution previously in the well compared to the millimolars of the solution that it was switched out for daily. As seen in Figure 1, as millimolars of the solutions increased, the rate of development decreased. After forty-eight hours, the zebrafish in the 0 mM (control) solution were 40% hatched, the zebrafish in the 30 mM solution were 50% hatched, the zebrafish in the 100 mM solution were 33% hatched, and the zebrafish in the 300 mM solution were 10% hatched. The chi square formula is $\frac{(O-E)^2}{E}$. The chi square value was 4.02, the degree of freedom that was used was 3, and the critical value was 7.82. Because the chi square value was less than 7.82, the null hypothesis was accepted.

Table 1: Number of Zebrafish Hatched and Alive

Amount of Ethanol in each solution.	Day 1 Alive	Day 1 Hatched	Day 2 Alive	Day 2 Hatched	Day 3 Alive	Day 3 Hatched	Day 4 Alive	Day 4 Hatched	Day 5 Alive	Day 5 Hatched
0 mM	10	0	10	0	10	0	10	4	10	10
30 mM	10	0	8	0	8	0	8	4	7	8
100 mM	10	0	9	0	9	0	9	3	9	9
300 mM	10	0	10	0	10	0	10	1	10	10

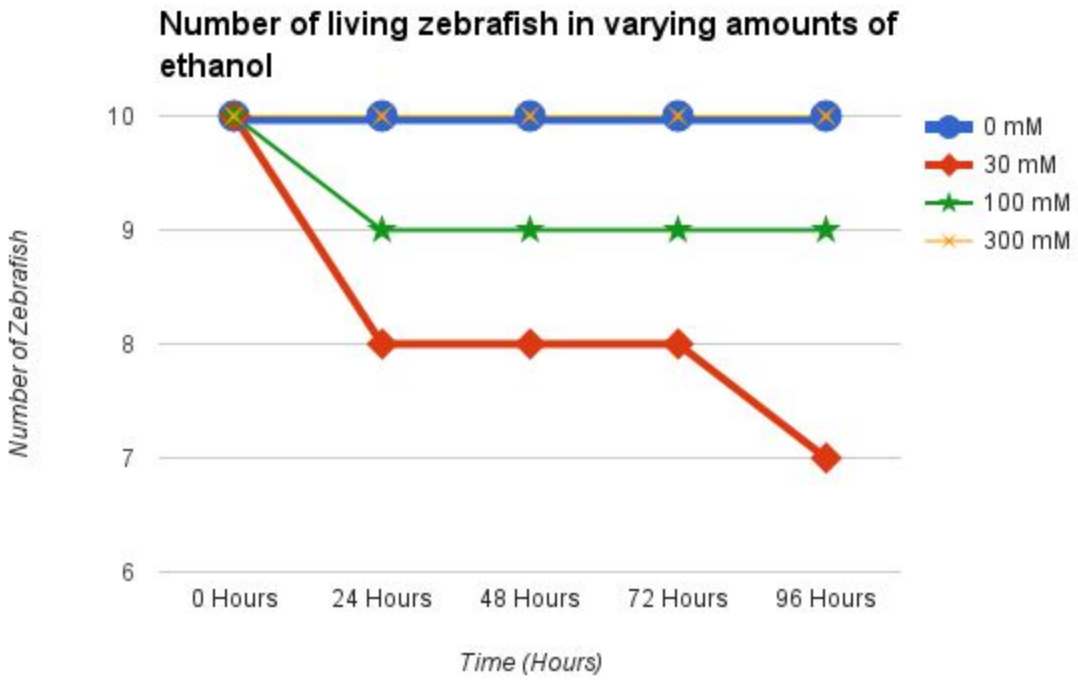
In Table #1: Number of Zebrafish Hatched and Alive, the results seen are a digital recreation of the actual sheet used to record some of the data gained from this experiment. It is seen that the wells containing the 0 mM and the 300 mM solutions had none of the zebrafish inside them die. The wells containing the 30 mM and the 100 mM solutions had a few zebrafish die. These results are also shown in Figure #1 and Figure #2.

Figure #1



From the results shown from Figure 1, it can be seen that the zebrafish in the control solution hatched the fastest, then the second fastest to develop was the 30 mM, then the 100 mM, and last the 300 mM even though all of them that were in the wells were hatched after seventy-two hours, there was variation between the different solutions after forty-eight hours which tells us the order of fastest to slowest development.

Figure #2



In Figure 2, you can clearly see that there were a few zebrafish deaths in the wells containing the 30 mM and the 100 mM solutions. Also in the 30 mM well there was one zebrafish that didn't die right away, but came out of the egg with a broken tail. The 0 mM (control) and 300 mM lines are merged because neither one of those wells had a zebrafish die.

But during the testing, it was found that out of the four amounts of ethanol (0 mM, 30 mM, 100 mM, and 300 mM), that the zebrafish in the 0 mM (control) solution (the control) four hatched after forty-eight hours, which is the same as the 30 mM solution (two were dead), the 100 mM hatched three zebrafish (out of nine) after forty-eight hours, and the 300 mM hatched only one after forty-eight hours. Then after the seventy-two hours, all ten were hatched in 0 mM (control), all eight were hatched in the 30 mM well, all nine were hatched in the 100 mM well and nine were hatched in the 300 mM well (out of ten). Then after ninety-six hours, all ten were hatched in the 0 mM (control) well, seven out of seven were hatched in the 30 mM well, nine out of nine were hatched in the 10mM well and all ten hatched in the 300 mM well.

Discussion:

The results almost support the hypothesis, which was that if zebrafish were put into different amounts of ethanol, then zebrafish in the wells with a higher concentration of ethanol would develop slower than those in lesser amounts of ethanol. Even though the results of the Chi square ($\frac{(O-E)^2}{E}$) were not high enough to be statistically significant (null hypothesis proved at three degrees of freedom, critical

value of 4.02), there was a 73.37% chance that something was going on. That was including the results of the final day where the effects of the ethanol on development were negligible compared to earlier days what was seen. This is important because zebrafish and humans react the same to a lot of materials so there is a very good possibility that there is a correlation between ethanol and slower development.

In Figure 2, it showed the zebrafish deaths, and it just shows that there were some factors that could have altered the data in Figure 1. However, it was speculated that the zebrafish in the 300 mM well must have been some very healthy embryos at birth, since none died, which was surprising. What was also perplexing was that two zebrafish died in the 30 mM, and one died in the 100 mM. From the research done about ethanol, the zebrafish in the 300 mM well should have had more dead zebrafish than any of the other wells, which was not the case in this experiment. In Figure 1, it can be seen that no zebrafish hatched until about 24 hours, which then 0 mM group had the most hatched in the fastest time from the starting time. The 30 mM well came next, followed by 100 mM, but the 300 mM well took awhile longer to hatch most. However, it is shown all were hatched by 48 hours, which means all hatched within about 24 hours. This trend, because the 0 mM well zebrafish hatched the most first, 30 mM probably second, 100 mM probably third and 300 mM probably last (Because some in 30 mM and 100 mM died so couldn't have been known if any wouldn't have hatched) supports the hypothesis that ethanol affects development of the zebrafish. It does so by slowing the development like how ethanol affects newborn babies by giving them "Brain damage and problems with growth and development" Alcohol during pregnancy. (2016, April). Retrieved January 12, 2017, from Marchofdimes.org.

The main focus of this experiment was on the development of the fish; basically when the zebrafish hatched, how many hatched on a certain day, and which solution had the most zebrafish hatch the fastest. The hypothesis was that if the zebrafish were in a higher concentration of ethanol then the longer it would take for them to develop. There is research that shows that ethanol in children can be harmful to their development. "Brain damage and problems with growth and development (Alcohol During Pregnancy. (2016, April). Retrieved from Marchofdimes.org)." Because of this it can be assumed that ethanol would be harmful to zebrafish embryos as well. But during the testing, it was found that out of the four amounts of ethanol (0 mM, 30 mM, 100 mM, and 300 mM), that the zebrafish in the 0 mM solution (the control) four hatched after forty-eight hours, which is the same as the 30 mM solution (two were dead), the 100 mM hatched three zebrafish (out of nine) after forty-eight hours, and the 300 mM hatched only one after forty-eight hours. Then after the seventy-two hours, all ten were hatched in 0 mM, all eight were hatched in the 30 mM well, all nine were hatched in the 100 mM well and nine were hatched in the 300 mM well (out of ten). Then after ninety-six hours, all ten were hatched in the 0 mM well, seven out of seven were hatched in the 30 mM well, nine out of nine were hatched in the 10mM well and all ten hatched in the 300 mM well.

There is a possibility that there were some errors in the experiment that was conducted. Some of these could include that there could have been a larger sample size, so that the data could have been more accurate because the outliers would have less of an impact on the data as a whole. Another solution to the larger impact of outliers on the data could be repeating the experiment multiple times so that the outliers couldn't impact the final data as much. Also, if the pipettes used to measure 1ml of each solution was measured by what looks like 1ml, but there could be discrepancies in that because there could have been slight errors by the manufacturer that would throw off the measurement which could be fixed by

purchasing higher quality pipettes or ones that have been tested on to make sure that the results are exact. There could also be human error in measuring that exact amount of liquid, which could be fixed by checking the vision of the testers or by making sure that the testers know how to perfectly measure the liquid in the pipette. Both of these could also be fixed by using a machine to measure the exact amount of liquid and pouring it into the well to make sure that the amount of liquid in each well was exactly 1ml. There could have also been discrepancies in the ways that the testers replaced the liquid that was in the wells, this could also have left some of the old liquid in the well which could have change the results by having too much liquid in one well. There could be differences in the times that the zebrafish were out of the solution, as the time it took to replace the liquid was not the same every time it was replaced, leaving the fish out of the solution for different times. This could possibly affect the development of the fish. These could also be fixed by a machine that was made to replace the solutions fast and take the same amount of time every time the solutions was replaced. The 30 mM solution hatched the fastest when percentages are used to say how many zebrafish were hatched. This result is skewed by two of the zebrafish dying in the 30 mM solution and therefore making the illusion that those zebrafish hatched the fastest if you judge development rate in percentage hatched. A question of further study would be how would minor changes in the amount of ethanol that the zebrafish were exposed to impact the development of the zebrafish, and would a pattern be shown?

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