The Impact of Ethanol on Developing Zebrafish

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Abstract

According to the CDC, in 2011 to 2013, 10% of pregnant women reported consuming alcohol in the last month (Tan, Denny, Cheal, et. al, 2015). This study aims to discover the effects of exposure to ethanol on development, using Zebrafish as a human stand-in. The study hypothesized that exposure to ethanol would lead to a higher mortality rate and visible deformations. One group of Zebrafish were exposed to 0.03% ethanol, and their appearance and behavior was compared to a group of Zebrafish in regular embryonic solution. This study found a correlation between exposure to ethanol and deformations, but found no trend between ethanol and a higher mortality rate. The findings may be useful in discovering the exact relationship between ethanol and development and finding better treatments for those with FASDs. (More specific statistic for beginning)

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Background Information

Why Fish?

Zebrafish (*Danio rerio)* are a tropical freshwater fish commonly used for modeling human diseases. Zebrafish are an ideal test subject due to the fact that they share 70% of our genes. In addition, zebrafish share many organs with humans, including the brain, pancreas, heart, intestine, eyes, bone, liver, kidney etc. (Burke, 2016). In humans, exposure to ethanol can cause fetal alcohol syndrome, which can affect the eyes, heart, kidneys and bones, all of which are found in zebrafish. Therefore, the effect of ethanol on these organs can be seen in zebrafish, giving people a better understanding of how ethanol affects development. (Medline Plus, 2019). While mice share the same class with humans, zebrafish are far cheaper and have many distinct advantages over mice. Zebrafish breed frequently, with each clutch between 50 and 300 eggs. In addition, the eggs are laid and fertilized outside of the mother, meaning it is easier to alter the eggs. Finally, zebrafish eggs are clear, which allows scientists to view development (Burke, 2016).

Why Ethanol?

In the United States, from 2011 to 2013, 10% of pregnant women reported consuming alcohol, and approximately 3% reported binge drinking in the past 30 days (Tan, Denny, Cheal, et. al, 2015). Drinking alcohol during pregnancy can result in fetal alcohol spectrum disorders, or FASDs. FASDs can result in abnormal facial features, smaller head size, below average height and weight, poor coordination, learning disabilities, intellectual disabilities, hyperactive behavior, and problems with vision, hearing, heart, kidney or bones. There is no cure for FASDs, and no medication approved to specifically treat FASDs (CDC, 2018). This study chose to focus on ethanol's impact on development, due to how prevalent this issue is in the United States. This

study hopes to better understand the effects of ethanol on prenatal development, and perhaps lead to the development of medication to specifically treat FASDs.

The Investigation

Zebrafish embryos were placed in 0.03% ethanol and their development, survival and hatch rate were compared to zebrafish embryos placed in pure Instant Ocean solution. The lab hypothesis predicts the zebrafish exposed to the ethanol solution will have a lower survival rate and visible deformations. The study found a correlation between exposure to ethanol and visible birth defects. In addition, it was found that the experimental fish had a higher survival rate.

Materials and Methods

Participants

In November 2018, two classes of Honors Biology students at Greendale High School tested the effects of certain chemicals on developing zebrafish and recorded the findings. The University of Milwaukee Wisconsin provided 120 zebrafish embryos for each lab group. Honors Biology teacher Mrs. Zientek teacher aided students in preparing the lab and incubated the zebrafish.

Materials

The lab required the use of a 12 welled falcon plate to store the zebrafish, a beaker for waste product, a beaker of Instant Ocean solution and a beaker for the ethanol solution. Additionally, an incubator was used to incubate the fish and a compound microscope was used to observe and count the fish. In addition, distilled water, Instant Ocean and ethanol were used to create the embryo mediums. Finally, the lab required the use of minimum bore and wide pore transfer pipettes to change the solution and to remove waste and dead fish (Petering, Berg, Tomasiewicz, et. al, 2018).

Design

The independent variable of this lab was the presence of 0.3% ethanol in the Instant Ocean solution. In this lab, the dependant variable was the number of Zebrafish that survived and hatched in each set of wells. The control group, which were placed in pure Instant Ocean solution, were wells A 1-4. The experimental group, which were placed in Instant Ocean solution mixed with ethanol, were wells B 1-4 and C 1-4. The sample size was approximately 120 Zebrafish, and the controls included the use of Instant Ocean solution, incubation at 28.5℃ and the removal of dead embryos and waste product every 24 hours for the 96 hours post fertilization. In addition, the embryos were placed in the wells at 24 hours post fertilization.

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Procedure

Day 1

1.) Fill the one row of wells of the plate with 1 mL of Instant Ocean solution using the disposable pipette.
2.) Fill the remaining wells with the appropriate ethanol stock solutions (Insert % ethanol solution). Divide the embryos so there are approximately 10 embryos in each well.
3.) Label the plate on the student data sheet
4.) Record exact numbers of live embryos on student data sheet.
5.) Observe your embryos under the dissecting microscope. Record observations on student data sheet
6.) Place each plate in the 28.5℃ incubator overnight.

Day 2

7.) Remove plate from incubator.
8.) Remove dead embryos from plate using the disposable pipette. Squirt dead embryos into waste beaker. Be careful to only remove the dead embryos.
9.) Count remaining embryos, hatched fish, and record in data table.
10.) Remove ethanol stock solutions from each well of the plate
11.) Replace the ethanol stock solutions with the appropriate fresh ethanol stock solution using a clean pipette each time.
12.) Remove Instant Ocean solutions from each control well of the plate
13.) Replace the Instant Ocean solutions with fresh Instant Ocean solution using a clean pipette each time.
14.) Place plate under dissecting microscope and record observations on student data sheet. Note/describe any developmental markers and abnormalities. Repeat for all wells

15.) Return the plate to the appropriate 28.5°C incubator.

Day 3

16.) Repeat Day 2 work and observations. Record all data.

Day 4

17.) Repeat Day 2 work and observations. Record all data.

18.) Place all embryos and fish in waste container. Your teacher will properly dispose of the organisms

19.) Use Google Spreadsheet to calculate average survival and hatch rate

20.) Use Graphpad to calculate p-value for statistical significance (Petering, Berg, Tomasiewicz, et. al, 2018).

Results

Summary of Results

The lab showed a trend between the hatching/survival rate and the presence of ethanol. Wells B 1-4 and C 1-4, which were exposed to 0.3% ethanol, had a greater survival and hatch rate, than that of the control group (wells A 1-4), which were kept solely in Instant Ocean solution.

Qualitative Data

Figure 1  Experimental 0.03% Ethanol - 96 hpf	Figure 1 shows a Zebrafish from the experimental group with an enlarged yolk sac and what appears to be an enlarged heart.

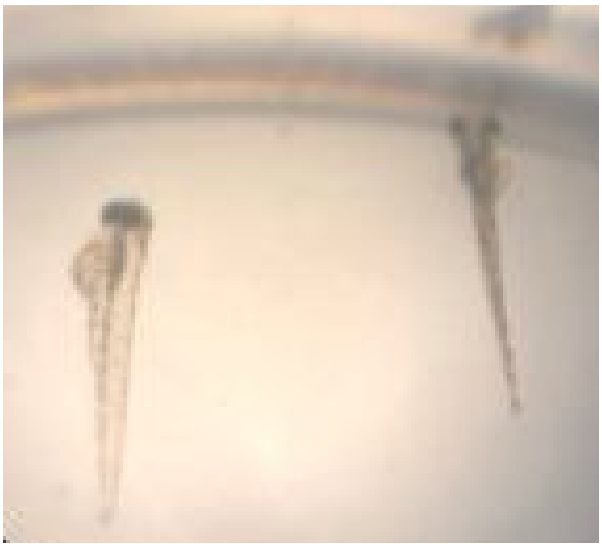
Figure 2  Control - 96 hpf	Control fish move quickly and respond to stimuli.

Figure 3  Experimental 0.03% Ethanol - 96 hpf	Figure 3 shows certain experimental fish appeared to have hatched before developing fully, lacking eyes and pigment.

Figure 4  Experimental 0.03% Ethanol - 96 hpf	Figure 4 is another example of the non-fully developed experimental fish.

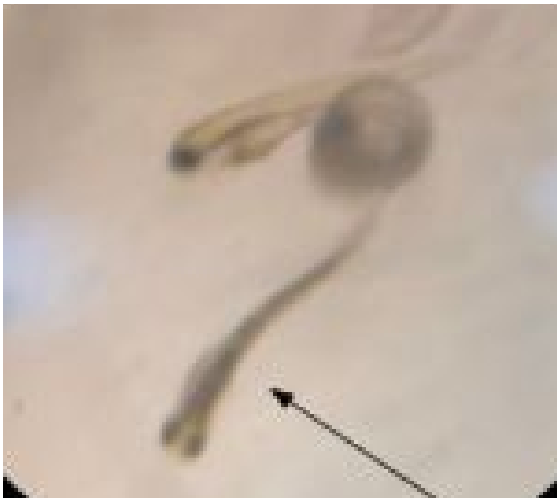
Figure 5  Experimental 0.03% Ethanol - 96 hpf	Figure 5 shows that in addition to large sacks and premature hatching, certain experimental fish also demonstrates curved spines. Most experimental fish struggled to swim and could only twitch.

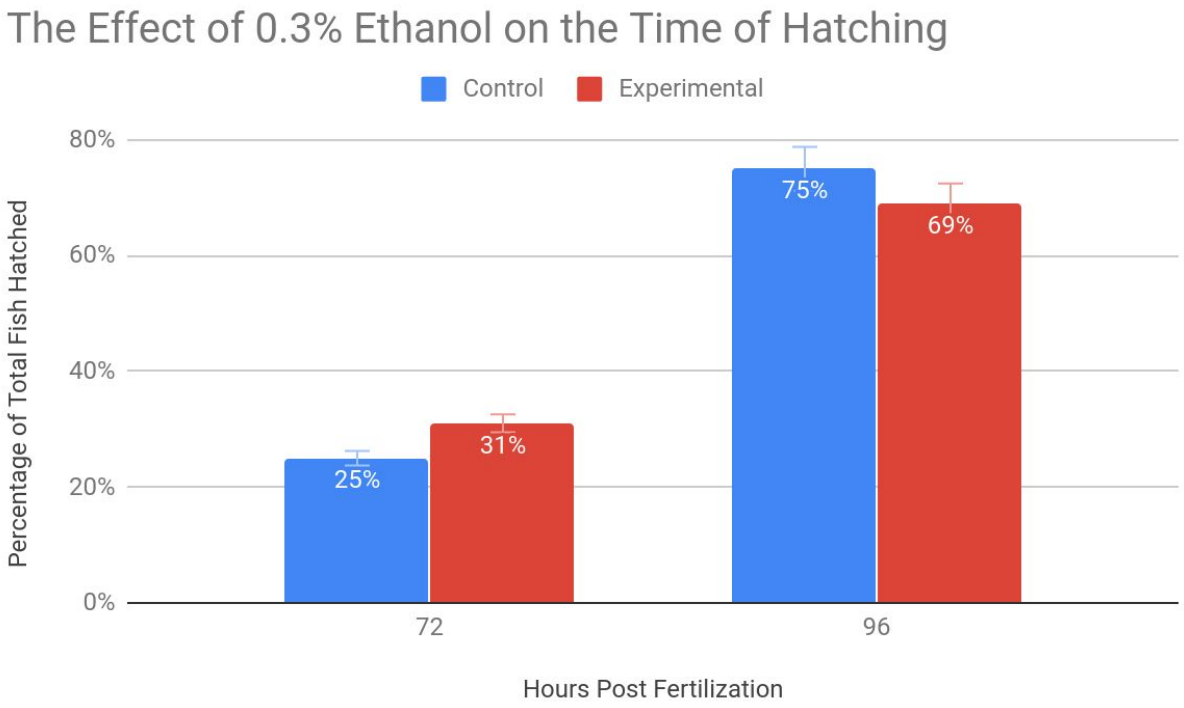
Figure 6

The Effect of Ethanol on the Survival and Hatch Rate of Zebrafish

	Control	Experimental
Survival rate	52%	75%
Percent hatched from total eggs (PHE)	39%	55%
Percent hatched from alive (PHA)	75%	74%

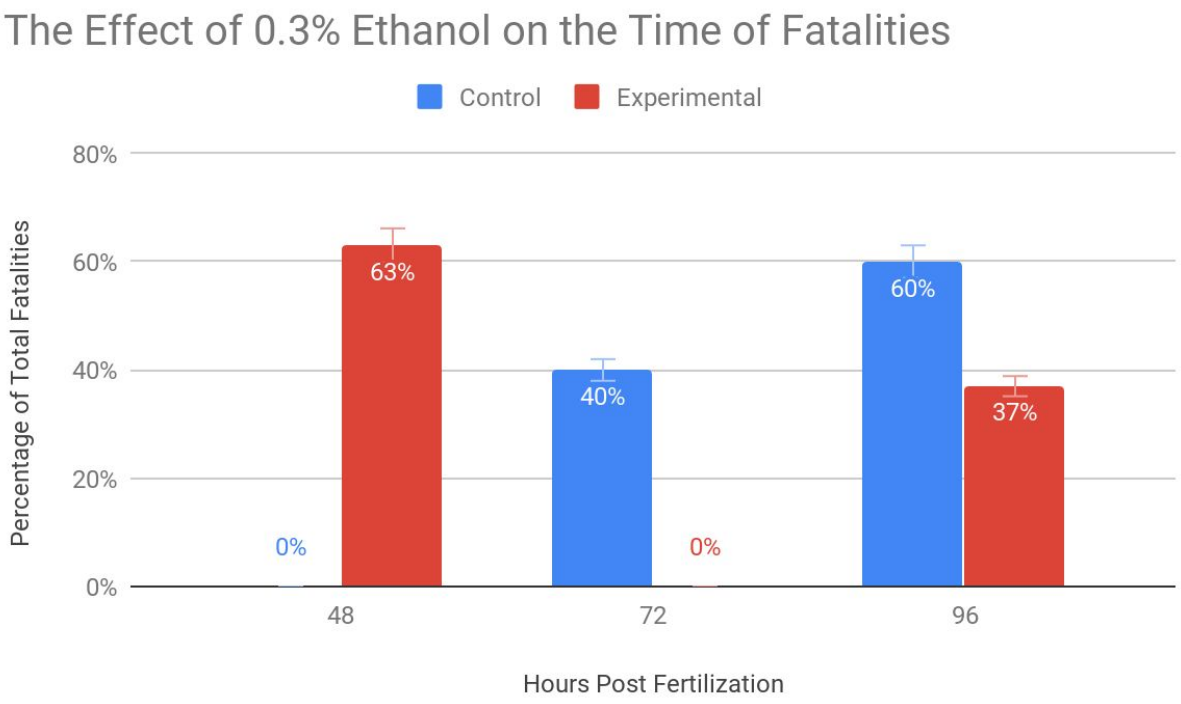
Figure 6 shows the difference in the survival rate, PHE, and PHA between the control and experimental group. There is a trend between the presence of 0.3% ethanol and a higher survival rate. There is not a statistically significant difference between the PHA from the control and experimental group. Additionally, there is a trend between the presence of 0.3% ethanol and a higher PHE.

Figure 7



This graph shows the relationship between the presence of ethanol and when hatching occurred. There is a trend between the presence of 0.3% ethanol and a higher percentage of hatching occurring at 72 hours post fertilization.

Figure 8



This graph shows when the fatalities in both groups occurred, organized in 24 hour increments. There is a trend between the presence of 0.3% ethanol and a higher percentage of fatalities occurring at 48 hours post fertilization. Additionally, there is a trend between the presence of 0.3% ethanol and a lower percentage of fatalities occurring at both 72 and 96 hours post fertilization.

Statistical Findings

Two tailed P value: 0.0183

Unpaired t-test: 2.3979

Figure 9

The statistical differences between the Control and the Experimental group

	Control	Experimental
Mean	0.52	0.75
SEM	0.09	0.05
SD	0.51	0.44
N	31	76

The unpaired t-test was because it is good at comparing the means of two groups where there are no shared test subjects. The Mean was used to compare the survival rates of the two groups. The SEM and the SD show how wide the peaks are of the two groups so one can see if the peaks overlap. This lab used the SEM because it compared the averages of two groups. As the p-value is less than 0.05, this data is considered to be statistically significant.

Experimental Design

The lab studied the effects of 0.3% ethanol on developing Zebrafish and compared it to the development of Zebrafish is pure Instant Ocean solution.

Design

The independent variable of this lab was the presence of 0.3% ethanol in the embryo solution, and the dependent variable was the survival and hatch rate. The controls included incubation at 28.5 degrees Celsius, use of some or all Instant Ocean solution in the embryo medium, removal of waste and dead embryos every 24 hours, and the placement of the embryos in the wells 24 hours post fertilization. The controls helped to maintain a similar environment for the embryos, so solely the effects of ethanol on development could be

observed. This lab has found that the presence of ethanol in the embryo solution led to an increased survival rate, and a decreased hatch rate.

Discussion

Importance of the Topic

The hypothesis at the beginning of this experiment was that exposure to ethanol would lead to a decreased survival rate and visible deformations in the zebrafish. However, the results of this study were mixed. This study found a correlation between exposure to ethanol and a greater survival rate, as seen in **Fig. 6**, which does not support the hypothesis. One possible explanation for this is ethanol's bacteria killing properties. In the control wells, there appeared to be bacterial growth, which may have contributed to some control fish mortalities. As seen in **Fig. 8**, the majority of the fatalities in the experimental wells occurred in the first 24 hours of being exposed to ethanol. It is possible that the experimental fish that survived the first crucial 24 hours in the ethanol solution had a better chance of survival due to ethanol's ability to kill off bacteria. However, as seen in **Fig. 1, 3-5**, there was a trend between exposure to ethanol and deformations, which supports the hypothesis. The experimental fish suffered from enlarged yolk sacks, curved spines and enlarged hearts. Many of the experimental fish struggled to swim and twitched uncontrollably. In addition, there appeared to be a correlation between exposure to ethanol and premature hatching, as several of the newly hatched experimental fish were not as developed as their control counterparts. All of these issues would drastically decrease the chances of long term survival. This leads this study to infer that exposure to ethanol leads to impaired and/or mutated development and a decreased chance of survival.

Sources of Error:

As with any study, there were possible sources of error, such as bacterial growth in the wells. In addition, this lab lacked a tool for precise extraction of dead embryos and waste product, which may have led to live embryos being removed accidentally. This also made it difficult to get an equal number of embryos into each well. Ideally, this lab would have spanned

a greater number of days to observe how the ethanol impacted the overall life span, eating, swimming and reproduction. In addition, this lab would have employed the use of a micropipette, had a greater sample size, and would have performed a post-mortem dissection to observe the effects of ethanol on the vital organs.

Importance of the Findings

As seen in the Background Section, the results of exposure to ethanol during pregnancy can be disastrous. With a terrifyingly high percentage of pregnant women drinking alcohol, it is imperative that society becomes more aware of ethanol's impact and how to treat it. As seen in **Why Fish,** zebrafish share many vital organs as well as 70% of their genes with humans, allowing scientists to view the effects of ethanol on these shared tissues, without exposing a human fetus to it. Understanding ethanol's impact can bring awareness, and hopefully decrease the number of pregnant women consuming alcohol. In addition, as seen in **Fig. 1, 3-5**, there is a correlation between ethanol exposure and birth defects. Further study of how and when ethanol impacts tissues could lead to understanding when most mutation occurs and perhaps lead to a medication to prevent it. Further study of how ethanol impacts development could lead to the development of drugs to specifically help children with FASDs. Overall, further study of the correlation between ethanol exposure and deformities is important to better understand FASDs and to hopefully find better treatments.

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