The Effect of Ethanol on Zebrafish Embryonic Development

Ellie Roder

Greendale High School

Abstract

This experiment was designed to investigate the effects of ethanol on zebrafish embryos, in order to predict the effect of ethanol on human embryos. The zebrafish embryos were exposed to ethanol at concentrations of 0.03%, 0.1%, and 0.03%, as well as a control treatment as a means of comparison. The embryos were observed for 120 hours post fertilization. The findings showed a 70% (+/- 15.3%) difference in percent of zebrafish surviving after 24 hours between the control treatment and the 0.3% ethanol solution. Statistically significant evidence was also found to support that zebrafish exposed to ethanol were less likely to hatch. Those embryos that did survive and hatch appeared more likely to have spinal deformities. Due to the similarities between zebrafish and human embryonic development, these findings could support the theory that alcohol consumption by pregnant women could have negative effects on the fetus. More studies would need to be done, with more trials and different concentrations, to specify the exact effects as well as specifying which concentrations would cause harm.

Background Information

Ethanol & FASDs

Ethanol, also known as alcohol, was chosen because it is a prominent drug in our society, and is known to cause physiological problems in both adults who consume it and fetuses exposed to it during development (National, 2020). Ethanol's chemical formula is C_2H_5OH, and the OH group allows it to attract to proteins in cell membranes, altering the function of intramembrane proteins (National, 2020). Ethanol often targets cells in the organs of the digestive, renal, cardiovascular, and nervous systems (National, 2020). The change in function of these proteins causes diseases like cirrhosis and addiction.

Ethanol use during pregnancy significantly increases the probability of miscarriage or birth defects, yet 7.6% of pregnant women in the US report drinking alcohol throughout their entire pregnancy (Bernstein & Vorgias, 2019). Ethanol is more dangerous to fetuses than adults because, when introduced during fetal development, it injures organ systems as they are still growing (Brien & Smith, 1991). The effect of ethanol on surviving fetuses is known as a range of disorders called Fetal Alcohol Spectrum Disorders, or FASDs (Bernstein & Vorgias, 2019). These disorders affect about 2.4-4.8% of the population of the US (Bernstein & Vorgias, 2019). The most severe FASD is known as Fetal Alcohol Syndrome, or FAS (Bernstein & Vorgias, 2019). FASDs include three categories of symptoms, including decreased size, abnormal facial features, and disorders of the central nervous system (Bernstein & Vorgias, 2019). Different patients display different severity of symptoms, and those diagnosed with FAS have at least one symptom in each category (Ali et al., 2011). About 0.6 - 0.9% of Americans are diagnosed with FAS, but this number is higher in people of racial minorities and lower socioeconomic status, likely due to a higher alcohol prevalence due to inequality and poverty (Bernstein & Vorgias, 2019).

The symptoms of FAS include physical deformities as well as behavioral and mental issues (Committee, 2000). FAS often presents with craniofacial abnormalities like cleft palate (Committee, 2000). Children with FAS often display hyperactivity and impulsivity similar to children with ADHD and often develop secondary disabilities, like ADHD and other neurodevelopmental disorders, that decrease social functioning (Committee, 2000). Children with FAS also often display difficulty with coordination, learning, hearing, and speech (Committee, 2000). The negative effects continue throughout the affected child's life, as adults with FAS are more likely to have mental health disorders, drug addiction, and inappropriate sexual behavior (Barr et al., 1996).

The negative effects of ethanol on developing human fetuses cause FAS, but there are conflicting results on the mechanism of ethanol in the cells and how that results in harm. An experiment by Brien & Smith in 1991 found that ethanol suppresses fetal breathing, decreasing oxygen to the brain. In 2000, A study by Bittigau et al. showed that the cause of FAS was apoptosis of neurons. In rats, ethanol, inhibited NMDA glutamate receptors and activated GABAA receptors, resulting in signal cascades that responded with apoptosis of cells in the rat's forebrain (Bittigau et al., 2000). This neurodegeneration could also affect humans (Bittigau et al., 2000). Other studies, such as a behavioral study by Annan et al. in 2014, suggested that exposure to ethanol during development causes inhibition of the oxytocin signaling pathway in zebrafish, leading to difficulty with social behavior later in life. This is reflected in research of humans with FAS, as they often have decreased quality of life in adults with FAS based on categories like mental health, unemployment, and dependent living (Barr et al., 1996).

Why Zebrafish?

Zebrafish are very useful in scientific studies, as they are generally considered more ethical as well as effective (Burke, 2016). First, they are easier to use than many organisms

(Burke, 2016). Their small size means they don't take up much space and are easier to care for (Burke, 2016). Furthermore, their eggs develop outside of the mother's body and the eggs are translucent, so different developmental checkpoints can be observed easily (Burke, 2016). They develop over a relatively short period of time compared to mammals, so it is easier to perform multiple trials (Burke, 2016). Finally, their genome is relatively similar to humans' so findings are often applicable to humans (Burke, 2016). Due to all of these reasons, many researchers use zebrafish in preliminary studies of the effects of different chemicals on living organisms (Burke, 2016).

Zebrafish are often used as a baseline, so research done with zebrafish is very effective because it can be compared to previous studies with the same organisms (Burke, 2016). Comparison to similar research is an effective way to determine if one study has accurate results or if it is an outlier. The abundance of past research on the effect of ethanol on zebrafish improves the ability to confirm repeatability of this experiment's results. A 2011 study by Ali et al. researched in which time windows ethanol caused the most detriment zebrafish embryos. They found that ethanol significantly slowed neural development and showed similarities to FAS in humans, based on phenotypes and behavioral impairment (Ali et al., 2011). There are also studies on the effect of embryonic ethanol exposure on adult zebrafish (Annan et al., 2014).

Results from these studies can inform the hypothesis and setup of this experiment. Zebrafish were used specifically to test the effects of ethanol because there is a large spectrum of effects that ethanol has on zebrafish, including decreased size, unusual development of the eyes, skeleton, yolk sac, and brain, as well as increased mortality rate (Ali et al., 2011). These effects overlap with human FAS (fetal alcohol syndrome), showing that zebrafish are an ideal model for testing the effect of ethanol on fetal development and survival (Ali et al., 2011).

The Investigation

Zebrafish were used to test the effects ethanol has on embryo's vertebrae development. Further studies would be needed to confirm the effects on humans specifically. The effects that ethanol has on developing fetuses are known to be harmful, and this study aimed to confirm what effects are seen using zebrafish in order to have an ethical experiment. The hypothesis predicted that as ethanol concentration increased, overall health of the zebrafish would decrease, including a lower survival and hatch rate, because the ethanol chemical disrupts normal function of their developing organ cells.

The experimental results showed that exposure to 0.3% ethanol solution decreased the survival and hatch rate of the zebrafish significantly. The effects of 0.1% ethanol solution were not shown to be significant over the 120-hour time period, but in repetitions of this experiment, data could be recorded in other categories, like behavior and physical deformation. When exposed to 0.3% ethanol, 0% of the zebrafish survived past 120 hours post fertilization, as compared to 70% in the control group. The zebrafish in 0.3% ethanol solution were also less likely to hatch after 120 hours post fertilization, at 6.25% compared to 80%. It can be concluded from this data that ethanol is detrimental to the health and development of zebrafish embryos.

Materials and Methods

Participants

Random selection was utilized to choose 91 viable zebrafish embryos at 24 hours post-fertilization from the batch provided by the UW-Milwaukee School of Freshwater Sciences.

Materials

91 zebrafish embryos

1 3x4 well plate with lid

Fine tipped pipettes

Wide tipped pipettes

3 glass bottles

100 mL graduated cylinder

10 mL graduated cylinder

Dissection microscope

500 mL waste beaker

350 mL instant ocean solution

43 mL ethanol

Incubator

White piece of paper

Lined paper for data table

Pen

Camera

Design

A 5-day experiment was conducted in which newly fertilized zebrafish eggs were exposed to ethanol. There were three trials of each treatment group, including a control with Instant Ocean solution and three different treatment groups, including: 0.03% ethanol, 0.10% ethanol, and 0.30% ethanol. Solution was changed and dead fish were removed daily, observing their development and recording quantitative data on hatch date and survival. When not being observed, the embryos were kept in an incubator to ensure that the temperature and light variables were controlled.

Procedure

1. Mix 97 mL Instant Ocean solution and 3 mL of 10% ethanol to make 100 mL 0.03% ethanol solution.
2. Place in a glass bottle and screw on the lid.
3. Repeat steps 1-2 with 90 mL Instant Ocean solution and 10 mL of 10% ethanol to make 100 mL of 0.1% ethanol solution.
4. Repeat steps 1-2 with 70 mL Instant Ocean solution and 30 ml of 10% ethanol solution to make 100 mL of 0.3% ethanol solution.
5. Use a wide-tipped pipette to take in 6-10 embryos that are 24 hours post-fertilization, taking care not to allow them all the way up into the bulb. Embryos obtained from UW-Milwaukee Freshwater Sciences Department.
6. Place in the first well, named well #1A of a 3x4 well plate.
7. Repeat steps 5-6 until all wells are full.
8. Check that all embryos are alive by placing a piece of white paper under the well plate. An opaque egg indicates that the embryo is not viable. A dissection microscope can also

be used to determine which embryos are alive. With the light of a microscope, deceased eggs will appear black or dark grey in color.

9. Use a wide-tipped pipette to move any dead embryos into the waste beaker.
10. If the number in a well decreases to below 6 embryos during the process of step 9, use the wide-tipped pipette to replace until there are at least 6, but no more than 10, embryos per well.
11. Fill each well in column 1 about ⅔ of the way full with instant ocean solution using a wide-tipped pipette.
12. Fill each well in column 2 about ⅔ of the way full with 0.03% ethanol solution using a wide-tipped pipette.
13. Fill each well in column 3 about ⅔ of the way full with 0.1% ethanol solution using a wide-tipped pipette.
14. Fill each well in column 4 about ⅔ of the way full with 0.3% ethanol solution using a wide-tipped pipette.
15. Record the number of embryos in each well in a data table as a base measurement at 24 hours post-fertilization.
16. Place cover on well plate.
17. Place well plate in incubator for 23 hours.
18. After 23 hours, remove well plate from incubator and observe embryos through a dissection microscope, taking photos of at least one well plate in each treatment as well as any unusual embryos.
19. Use white paper to view each well, recording number alive, number hatched, and number dead in each well. Specify which are embryos that have died and which are hatched zebrafish that have died.

20. Remove any dead embryos using a wide-tipped pipette, taking care to not remove any living zebrafish, and place in waste beaker.
21. Use the fine-tipped pipette to remove all water from each well.
22. Repeat steps 11-21 until 21 hours post-fertilization.
23. After all final numbers are recorded, remove all water and embryos from the well plate using a wide-tipped pipette and place in petri dish.
24. Dispose of the zebrafish by freezing.
25. Use a Graph Pad two-tailed T-Test to analyze significance of the data (Graph Pad, 2019).

Results

Summary

The independent variable of ethanol concentration was manipulated to determine the effect of ethanol concentration on dependent variables of survival, hatching time, and development of the spine. Variables like age, temperature, amount and type of liquid, frequency of replacing waste liquid, and amount of light were controlled. Zebrafish embryos raised in experimental concentrations of 0.03%, 0.1%, and 0.3% ethanol, along with a control treatment of just Instant Ocean solution, were tested to determine the effects of ethanol on zebrafish development. Data was taken every 24 hours recording the dependent variables of percent surviving, percent hatched, and qualitative data was taken observing physical defects in the spines of the zebrafish. It was found that 0.3% concentration of ethanol decreased survival rate and decreased percentage hatched, and 0.03% and 0.1% ethanol caused visible physical defects in the surviving zebrafish.

Percent Survival

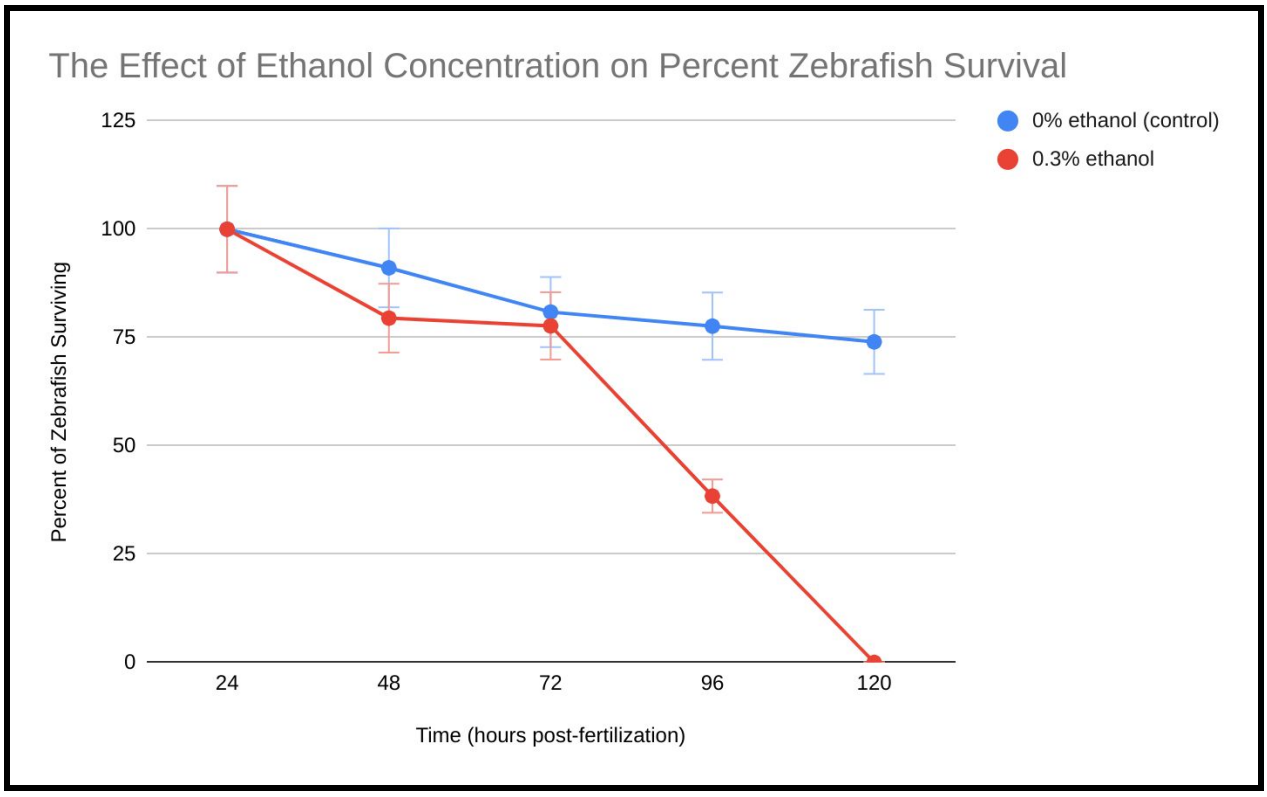

Figure 1: The blue line represents the average percentage surviving in the control solution of instant ocean solution at each 24-hour time point. The red line represents the average percentage surviving in 0.3% ethanol solution. Data was recorded at 24 hour intervals over the 96 hour period of observation. Range of n = 36-60 embryos per treatment, as experiment was repeated using the same procedure by Julien, O. & Trinkner, G. in 2019.

Zebrafish were determined to survive when they were not opaque under the dissection microscope. Figure 1 shows that although survival is similar between the control and 0.3% ethanol zebrafish at the start, after 72 hpf, the percentage of surviving zebrafish exposed to 0.3% ethanol solution decreased to a lower level than the percentage of surviving control zebrafish. There was not a statistically significant difference before 96 hours post-fertilization but after 120 hpf, the average percent surviving in the control solution was 70% +/- 15.3% and in the 0.3% ethanol solution the average was 0% +/- 0% ($p = 0.0102$) (GraphPad, 2019). This low

p-value means that the results are statistically significant, so it can be concluded that ethanol at a concentration of 0.3% from 24 hpf to 120 hpf does decrease likelihood of survival for zebrafish embryos.

Hatching and Development Time

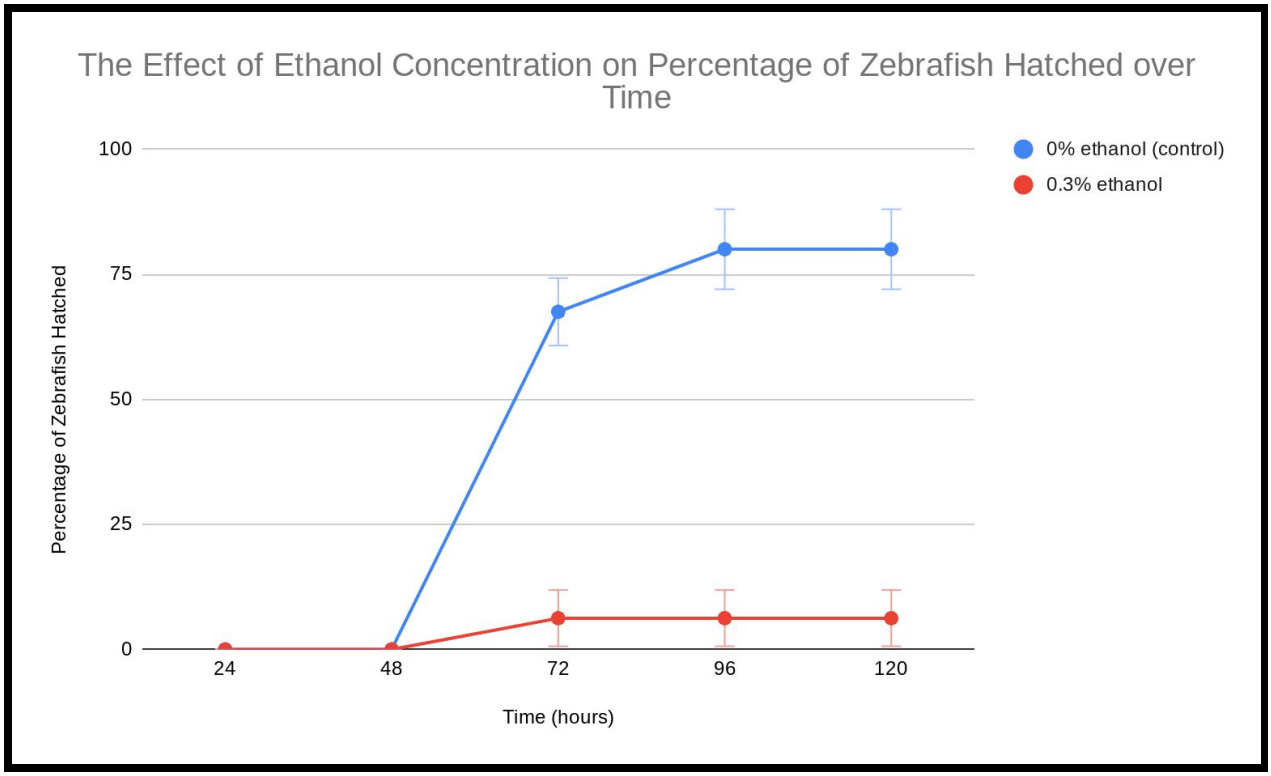

Figure 2: The blue line represents the average percentage hatched in the control solution of instant ocean solution over time. The red line represents the average percentage hatched in 0.3% ethanol solution. Data was recorded at intervals of 24 hours over the 96 hour observation period. Range of n = 36-60 embryos per treatment, as experiment was repeated using the same procedure by Julien, O. & Trinkner, G. in 2019.

Zebrafish were defined as hatched when they were fully out of the egg sac and a broken egg sac was floating in the water. If the zebrafish died after hatching, it was still counted as hatched even in the days after removal. The hatch data over the course of the experiment can be viewed in Figure 2. After 120 hours, the percentage of zebrafish hatched in control solution was 80% +/- 11.457% and the percentage hatched in 0.3% ethanol solution was 6.25% +/- 3.608% (p =

0.0009) (Graph Pad, 2019). This data was extremely statistically significant, supporting the prediction that as concentration ethanol increases to 0.3%, the percentage of zebrafish able to hatch decreases dramatically.

Spinal Development

Figure 3: Normal hatched zebrafish embryo in control solution (96 hours post-fertilization) viewed through a dissection microscope. This zebrafish was determined to be healthy, without any spinal defects.

Figure 4: In the red circle is a hatched zebrafish with deformed spine in 0.1% ethanol solution (96 hpf) viewed through a dissection microscope. Its spine was determined to have about a 90-degree angle bend.

Some zebrafish had spinal defects, otherwise known as scoliosis (see Figure 4 as compared to Figure 3). Zebrafish with scoliosis were only found in the 0.03% and 0.1% ethanol wells. No abnormalities were found in hatched zebrafish in the control wells. In the 0.3% ethanol wells, only two survived to hatching but neither had a visible spinal defect. No quantitative data was recorded, but if this experiment was repeated, number of fish with spinal defects (specified by degree bend in the spine) could be recorded for a more significant result.

Sources of Error

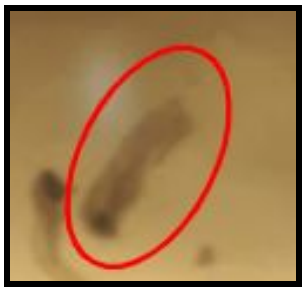

Figure 5: In the red circle is a hatched zebrafish that died of fungal infection (72 hpf) viewed through a dissection microscope.

Some hatched zebrafish died due to fungal infections (see Figure 5), so the results were not completely accurate because some deaths were not related to ethanol. These deaths were still counted in calculations for survival, so that could skew the results. However, this error did not completely disqualify the results of the experiment because not all deaths were caused by fungus, and fungus was just as likely to harm fish in the control treatment as it was to harm fish in any of the experimental treatments. To make future experiments more accurate, this error can be prevented by increasing the amount of methylene blue, a chemical that kills fungus in the water without causing any known harm to the zebrafish. This way, any deaths would be confirmed to be from the effects of ethanol, not any other cause or variable, so the results would be more dependable.

Discussion

Ethanol's Significance

This experiment aimed to show what effects varying concentrations of ethanol had on developing embryos, as those embryos are similar to developing human embryos. The results reinforced the hypothesis that ethanol is detrimental to the health of a developing embryo. It showed that, in the presence of ethanol, the zebrafish were less likely to survive past 96 hpf, less likely to hatch. It can also be inferred that ethanol causes harm to the spinal development, although the results of this experiment were not focused on that effect, so more intensive research would need to be done.

A widespread problem exists in humans since many often consume the addictive drug known as alcohol, which is also known by its chemical name ethanol (National, 2020). A correlation between alcohol consumption in pregnant mothers and birth defects has been confirmed (Barr et al., 1996), and these defects are diagnosed as FASDs. It is important to study exactly how ethanol affects embryos in order to protect human embryos from death or future disease, but this research is limited due to ethical dilemmas in experimenting on human brains (Bernstein & Vorgias, 2019).

Many animal models, including zebrafish and rats, have demonstrated that ethanol causes fetal harm (Diaz et al., 2012). Testing on human fetuses is unethical, so surveys and statistical counts, while flawed because variables are harder to control and they rely on self-reporting, have also been conducted to confirm that ethanol consumption increases likelihood of FASDs (Bernstein & Vorgias, 2019). About 7.6% of women report drinking after finding out they are pregnant, and these mothers have a much higher probability of miscarriage than mothers who do not (Bernstein & Vorgias, 2019). These mothers are also more likely to give birth to an infant with FASDs (Diaz et al., 2012). Many women stop drinking after finding

out they are pregnant, but this often occurs six weeks post fertilization or later, and can still result in FASDs and a higher rate of miscarriage (Bernstein & Vorgias, 2019). This is supported by the 2011 study by Ali et al., which showed that behavioral impairment and physical defects were most common in zebrafish exposed to ethanol during the earlier stages of development.

Importance of the Results

The findings of this experiment supported the hypothesized claim that ethanol at concentrations of 0.3% will harm the health of the zebrafish. Statistically significant data showed that in zebrafish, ethanol can cause a lower survival rate and a lower likelihood of hatching. Observations also suggested that ethanol causes a higher rate of physical defects in the spine.

The survival results, which were statistically significant (Graph Pad, 2019), indicated that survival of zebrafish after 96 hpf is lower when exposed to ethanol. Ethanol decreased birth rate as well as survival of hatched zebrafish, further showing that ethanol increases infant mortality and likely causes similar results in humans. These results reflect results of the 2011 experiment conducted by Ali et al., which showed that when exposed to ethanol at the earlier stages of development, the mortality rate of zebrafish increased to 88%. This zebrafish model can be used to reflect human survival, providing more insight into the higher infant mortality rates in communities with a higher prevalence of alcohol (GBD, 2018).

The hatch data, which was also statistically significant (Graph Pad, 2019), showed that zebrafish are less likely to hatch when exposed to 0.3% ethanol solution. This supports the hypothesis that ethanol increases hatching time, therefore representing that the presence of ethanol slows development. This is a form of harm to the developmental health of the zebrafish, further bolstering the stated hypothesis. Therefore, it can be concluded that concentrations of ethanol 0.3% or greater harm the health of developing zebrafish.

Finally, more data could be recorded to triangulate data with all three categories of FAS symptoms, including facial abnormalities, disorders of the central nervous system, and decreased size (Bernstein & Vorgias, 2019). The zebrafish exposed to 0.1% and 0.03% ethanol did not show significant effects, but if more data was recorded, this lower concentration could have yielded significant results, because when exposed to less ethanol, zebrafish have higher survival rates, and phenotypic and behavioral symptoms are only diagnosed in embryos that survive (Committee, 2000). When exposed to less ethanol, zebrafish generally have higher survival rates (Annan, et al., 2014). These surviving zebrafish could, however, have defects shown in differing phenotypes and behavior (Ali et al., 2011). Addition of these categories of data would add to the usefulness of the survival and hatch results to further confirm the effects of ethanol on developing zebrafish.

Although quantitative data was not taken to prove the effect of ethanol on the spine, the results (shown by Figure 4) suggested that ethanol causes scoliosis. Repetitions of this study could record categorical quantitative data on the amount and degree of spinal bending. This would correspond with phenotypic differences, like facial abnormalities, in humans with FAS (Ali et al., 2011)

Similarly, behavior could have been studied to connect to the damage to the central nervous system in humans with FAS. Research from Blaser et al. in 2006 used video tracking to study the effects of ethanol on zebrafish behavior in a predator-prey environment. Their findings suggest that ethanol impairs behavior of adult zebrafish and hinders survival (Blaser et al., 2006). Ethanol can also increase anxiety and unusual social behavior in adult zebrafish which were exposed to ethanol during development (Annan et al., 2014). If the experiment was longer and recorded data on behavior, the exposure of zebrafish embryos to ethanol could have had similar results.

Finally, size could have also been measured by measuring length and width of hatched zebrafish every 24 hours after exposure. Humans with FAS display decreased growth rate (Bernstein & Vorgias, 2019). Measuring size of the zebrafish could add substance to the argument that ethanol causes FAS-like symptoms in zebrafish as well as humans.

Relation to Humans

The findings in the current study may be extendable to a conclusion about the harm caused to human embryos when exposed to ethanol in the womb. However, this statement will need more extensive research as there may be differences in effect and concentrations in the health of human fetuses, which take longer to grow and become much larger with more complex organ systems. New claims are suggesting that there is no safe concentration, as any concentration of ethanol being added to the body can denature the cell's proteins (National, 2020), inhibit signaling pathways in the brain (Annan et al., 2014), and inhibit brain development, depending on when ethanol is introduced (Ali et al., 2011).

Although decreased concentrations of ethanol resulted in decreased negative effects, as little as 1 drink per day causes a significant increase in likelihood of FAS (Committee, 2000). Fetuses exposed to lower concentrations of alcohol are also more likely to develop FASDs than FAS, which still harm social behavior and physical health (Bernstein & Vorgias, 2019). There is no level of alcohol consumption in pregnant women that is confirmed to be safe for the fetus, so the American Academy of Pediatrics recommends abstaining from all alcohol consumption during pregnancy (Committee, 2000).

Compared to the control fish, zebrafish exposed to ethanol during development are less likely to interact socially with other zebrafish as adults, and they showed increased anxiety when exposed to new environments (Annan et al., 2014). The effects shown in zebrafish are also seen in humans, as about 80% of adults affected by FAS have unstable employment (Barr et

al., 1996), which could be caused by difficulty in situations requiring social interaction, like a stable career. Ethanol was also found to inhibit oxytocin production (Annan et al., 2014), and this knowledge helps in understanding why 90% of humans diagnosed with FAS also have mental health problems, often caused by central nervous system disorders, a part of FAS (Barr et al., 1996). The effects of lower concentrations of ethanol modeled by zebrafish could reflect how ethanol affects behavior and mental health of humans as well, showing why humans with FAS are negatively affected later in life.

The results of exposing zebrafish embryos to ethanol showed that ethanol is deleterious to fetal development, causing decreased survival and hatching. Those that survive will have decreased quality of life, and the effect of ethanol on zebrafish mirrors FAS, including decreased development of the central nervous system, abnormal structural growth, and decreased growth rate (Ali et al., 2011). More research is needed to confirm how ethanol affects zebrafish and to connect that to its effects on humans, but it is clear from the results of this experiment that ethanol harms embryonic development in zebrafish, and exposure should be avoided during human fetal growth. This research can help to spread awareness of FAS as well as inform scientists about effects and mechanisms of FAS, which could potentially lead to better prevention and treatment for infants exposed to ethanol in the womb.

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