The Effects of Wine and Ethanol Solutions on the Physical Development of Zebrafish

Kelly Koerber

Union Grove Union High School

Abstract

The purpose of this lab investigation is to determine if a simulation wine solution has different effects than an ethanol solution on the physical development of zebrafish. The data and observations gathered during this experiment could help to determine if ethanol affects a fetus in the same way that a mock wine solution would. This could help paint a clearer picture of the effect of alcohol on the development of an embryo. *Danio rerio*, more commonly known as a zebrafish, was used as a model organism in this experiment. Multiple zebrafish embryos were divided into three different experimental groups: the control group ("Instant Ocean" Solution), the 100 mM ethanol solution group, and the simulation wine solution group. During this lab investigation, it was determined that the lowest percentage of hatchlings, the highest percentage of deaths, and the greatest number of deformities were found in the 100 mM ethanol solution. However, the simulation wine solution had the highest percentage of hatchlings, the lowest percentage of deaths, and a considerable number of deformities. Despite these statistical observations, a conclusive understanding of the differences between ethanol and wine solutions on embryonic development could not be made. This is due to the confounding variables encountered during this experiment and the high mortality in the control solution.

Introduction

It is a well-known fact that expecting mothers should not consume alcohol while they are carrying a child. A developing fetus receives all of the nutrients needed for growth and development from their mother. Thus, anything the mother eats or drinks -including alcohol- travels to the fetus through the umbilical cord (Alcohol Use in Pregnancy). Fetuses that develop in the presence of alcohol are at a higher risk of developing embryonic disorders. These disorders

include but are not limited to “abnormal facial features, small head size, shorter-than-average height, low body weight, poor coordination, and hyperactivity” (Alcohol Use in Pregnancy). Quite a few studies have been conducted to support the idea that ethanol, a type of alcohol, has a negative effect on the development of zebrafish. While this is very valuable information, mothers-to-be are not simply consuming an ethanol solution. They are consuming alcoholic beverages that have considerably more ingredients.

One commonly consumed alcoholic beverage is wine. Wine seems as though it would simply contain fermented fruit and some sugar, but wine contains many other surprising ingredients like potassium sorbate, calcium carbonate, sulfur dioxide, and powdered tannins (10 Ingredients You Probably Didn't Know Were in Your Wine). These substances seem like chemicals that should only be used in a laboratory setting; however, these ingredients are regularly used in the making of wine. This observation poses the question: does the experimentation of an ethanol solution truly epitomize the effect of alcohol on a developing fetus? There are many other components of wine that could lead to physical disorders during development. Therefore, to test this potential variable, a mock wine solution consisting of realistic amounts of ethanol, sugar, and calcium carbonate was tested.

Danio rerio, also known as a zebrafish, is an adequate species to use in this experiment because they are a model organism. A model organism is “a species that has been widely studied, usually, because it is easy to maintain and breed in a laboratory setting and has particular experimental advantages” (What are Model Organisms?). Zebrafish have a “high rate of reproduction, short generation time, rapid development, external fertilization, and translucent embryos” (Why Study Zebrafish?). Furthermore, zebrafish are an adequate species to test in this

experiment. Based on previous research and outside knowledge on this topic, it seems as though fetuses submerged in the simulation wine solution will develop more deformities and have higher mortality than the embryos in the ethanol solution. This is due to the fact that the wine solution contains both ethanol and calcium carbonate.

Materials and Methods

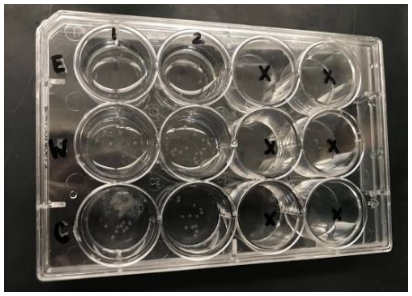
Three 1000 mL beakers, three 150 mL beakers, a stirring rod, disposable pipettes, 1000 mM ethanol stock solution, granulated sugar, $CaCO_3$ powder, embryo media, zebrafish embryos, scissors, a sharpie, a clean well plate, a dissecting microscope, and a compound microscope are needed in order to complete this lab.

In this experiment, equal concentrations of a simulation wine and ethanol solution were prepared. The ethanol solution was made by combining 100 mL of 1000 mM ethanol stock solution with 900 mL of Embryo Media. 500 mL of this diluted ethanol solution was measured and placed into a beaker labeled “Ethanol Solution,” and the other 500 mL was used to make the mock wine solution. For the wine solution, 0.2139 grams of $CaCO_3$ and 11.98 grams of sugar were mixed into the other 500 mL ethanol solution. This beaker was labeled “Wine Solution.” A 500 mL solution of Embryo Media was also made and poured into a beaker labeled “Control.” Once all of the required solutions were prepared, a well plate was labeled (Refer to *Figure* 1.). Next, approximately 10 embryos were pipetted into each well of the first two columns of the well plate (23 to 24 embryos per condition). A few embryos were placed under a compound microscope for initial observations of stages in development and physical deformities. After recording observations, the excess embryo media solution was pipetted out of the wells and into a beaker labeled “Waste.” Then, 2 mL of the corresponding solution was pipetted into the correct

well. It is important to note that the simulation wine solution should be stirred with a stirring rod before it is pipetted into the wells. The embryos were placed into an incubator that was heated to 28.5 °C.

The next day, the well plate containing the embryos was removed from the incubator. The number of unhatched, hatched, and dead embryos were counted and recorded using a dissecting microscope. Embryos that stood out from each condition were pipetted from the well and observed under a compound microscope. Pictures were also taken at this step. Once all observations were complete and all embryos were returned to their correct well, the day old solutions were pipetted from the wells into the waste beaker. In order to lessen the error of losing zebrafish embryos, a waste beaker was labeled for each solution. Thus, if an embryo was accidentally pipetted into the waste beaker, the embryos were not contaminated and could be pipetted back into their original well. In addition to the old solution, dead embryos and zebrafish were pipetted from the wells. Using different pipettes for each solution, 2 mL of fresh solution was pipetted into each well. Finally, the well plate was placed into the incubator. These steps were followed each day until the 102-hour mark.

Figure 1. Labeled Well Plate and Materials



As described in this procedure, the outcomes of this lab were measured by recording the number of unhatched, hatched, and dead zebrafish during each observational period. The data

that was collected was analyzed by comparing both the quantitative and qualitative results. The quantitative results were compared numerically by noting the differences in the number of hatched and dead zebrafish at 102 hours past fertilization. A calculation of percent difference was computed between the three different solution totals to find out the significance of the quantitative data. The qualitative data was used to compare both the overall difference in deformities and functional abilities of the zebrafish over the duration of the lab. Lastly, as with all labs, there are some safety concerns. The substances of concern are primarily calcium carbonate and ethanol. Calcium carbonate is an "eye, skin, and respiratory tract irritant and can cause central nervous system effects" (MSDS Calcium Carbonate). Ethanol is "flammable, toxic, and an eye and skin irritant" (Safety Data Sheet Ethanol). Therefore, it is important to follow safe laboratory practices while completing this lab.

Results

In this experiment, zebrafish embryos were observed over a 102 hour time period to determine if there were differences in development in embryos exposed to an ethanol or a simulation wine solution. Both the ethanol and wine solutions were made with 100 mM ethanol solution. The independent variable in this lab was the solutions that the experimenter chooses to submerge the embryos in (Instant Ocean [control] solution, an Ethanol solution, or a Simulation Wine solution). The dependent variable was the developmental reactions that the embryos had to one of the three solutions. This was measured quantitatively by the number of unhatched, hatched, and dead embryos and qualitatively by observations. The constants in this lab investigation were the type of well plate used, the amount of solution in each individual well, the incubation temperature, and the amount of light exposure. The control group was the embryos

that were only in the presence of Instant Ocean solution. Thus, these embryos can be used as a benchmark of comparison for the other solutions.

Table 1. Day 1 Raw Data

6 hpf	*Unhatched*	*Hatched*	*Dead*	*Total*	*Observations*
Control 1	10	0	0	23	All embryos placed into the wells seemed to be alive. The embryos were all clear and none of the embryos looked deformed or opaque. Therefore, the embryos were put into their correct solutions. The wine solution was a bit cloudy compared to the other solutions. This is likely due to the added $CaCO_3$. There was not much movement of the embryos at this stage.
Control 2	13	0	0		
Ethanol 1	12	0	0	23	
Ethanol 2	11	0	0		
Wine 1	10	0	0	24	
Wine 2	14	0	0		

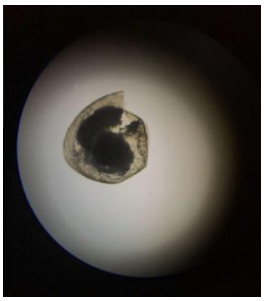
Table 2. Day 2 Raw Data

30 hpf	*Unhatched*	*Hatched*	*Dead*	*Observations*
Control 1	8	0	2	The dead embryos turned white while the living embryos looked clear.
Control 2	13	0	0	An alive embryo was viewed. The embryo had a tail, visible eyes, and a bloated, dark upper portion developing. (30 to 36 hpf)
Ethanol 1	11	0	1	The dead embryo was brown and visible broken.
Ethanol 2	8	0	3	An alive embryo was viewed. The embryo had eyes and a developing tail. There was some movement. (24 to 30 hpf)
Wine 1	8	0	2	The most movement while observing the embryos was found in the wine solution embryos.
Wine 2	13	0	0	An embryo with a brown spot was observed more closely. The brown spot was on the egg sac, and the embryo had most movement while viewing. (24 hpf)

***While extracting the waste solution from the wells, one live embryo from the Control 2 well, one live embryo from the Ethanol 1 well, and three live embryos from the Wine 2 well were accidentally pipetted with the waste solution into the waste beaker. Due to contamination, the embryos were left out of the experiment and were not pipetted back into their appropriate wells.*

*Alterations were made to the procedure -as described above- to ensure that no more embryos were lost in this fashion.***

Ethanol Well 2 *Control Well 2* *Wine Well 2* *Ethanol Well 2*



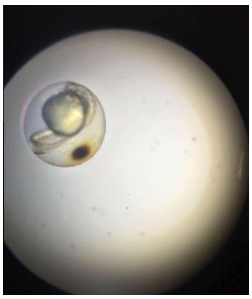
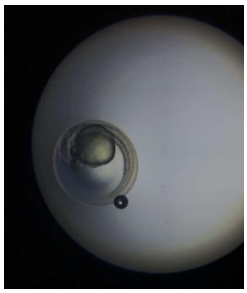
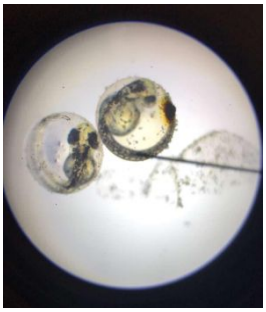
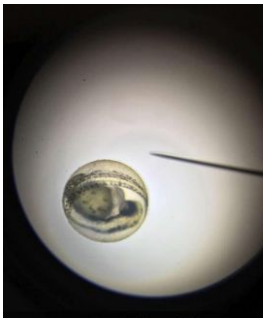
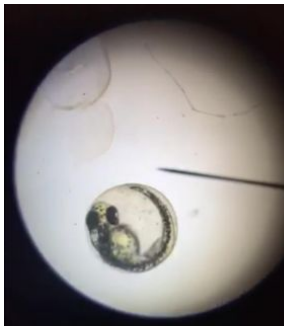



Table 3. Day 3 Raw Data

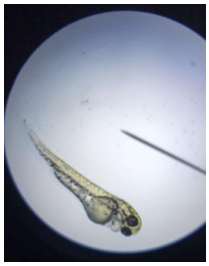
54 hpf	*Unhatched*	*Hatched*	*Dead*	*Observations*
Control 1	1	5	2	Could see circulation and heartbeat, but it was slower than Wine 1 heartbeat; eyes evident (48 to 60 hpf)
Control 2	8	3	1	Circulation is noticeable; movement is evident and the eyes are as well (48 to 60 hpf)
Ethanol 1	2	8	0	lots of spots; looks green/brown; eyes are evident and the belly is large compared to other embryos
Ethanol 2	2	4	2	Can see circulation; some movement is noticeable, but less than Wine 2 (36 to 48 hpf)
Wine 1	6	1	1	Could see circulation and heartbeat, but it was slower than Wine 1 heartbeat, eyes evident
Wine 2	6	3	1	Sugar crystals stuck to egg, green colored, and tail movement is evident (30 to 36 hpf)

Wine Well 2 *Ethanol Well 2* *Control Well 1* *Control Well 2*



Ethanol Well 1 *Wine Well 1*



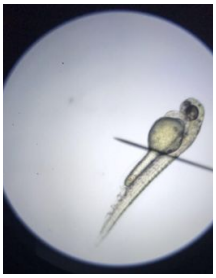
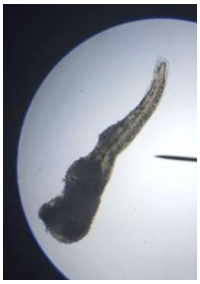


Table 4. Day 4 Raw Data

78 hpf	*Unhatched*	*Hatched*	*Dead*	*Observations*
Control 1	0	6	0	Longer than Wine, but about the same size as Ethanol, long fins, more active than other solution embryos (72 to 96 hpf)
Control 2	0	11	0	Deformed fish~possibly an outlier or caused by a fungus; normal development, very active, longer (72 to 96 hpf)
Ethanol 1	1	8	2	Dead fish were dark with many spots and had deformities; only unhatched embryo had deformed fish and was likely dead
Ethanol 2	0	6	0	Observed fish was long, yellow by the head, seemed further in development compared to Wine solution, and had developed fins (60 to 72 hpf)
Wine 1	0	7	0	Fish was not as long as Ethanol, fins were evident, some yellow on head and body (48 to 60 hpf)
Wine 2	0	11	0	Smaller than Ethanol, many brown spots, slightly deformed with yolk sac attached to the side rather than the bottom (36 to 48 hpf)

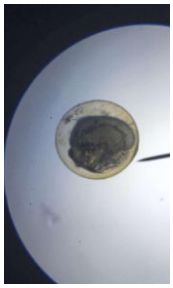
Control Well 2

Ethanol Well 1



Ethanol Well 1

Ethanol Well 1



Wine Well 2

Control Well 1

Ethanol Well 2

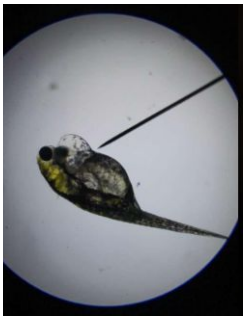
Control Well 2

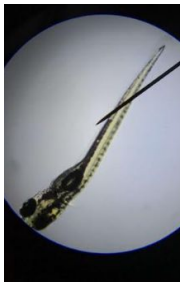
Table 5. Day 5 Raw Data

102 hpf	*Unhatched*	*Hatched*	*Dead*	*Observations*
Control 1	0	6	0	Long fish compared to other solutions as a whole, yellow coloring is not as vibrant (96 hpf)
Control 2	0	9	2	Dead fish: first was completely gray with spots, other was deformed and curled; live fish: not as yellow as previous day observations, eyes more developed with normal development (96 hpf)
Ethanol 1	0	7	2	Yolk sac attached to the side rather than the bottom, yellow, curled tail, little movement (60 to 72 hpf); long, yellow, evident fins, not much movement, sensitive to stimulus (72 to 96 hpf)
Ethanol 2	0	6	0	Yellow, spots, head developing into distinguished shape (96 hpf)
Wine 1	0	6	1	Curled tail, some yellow, little movement in solution (60 to 72 hpf); longer compared to the previous day, dark body, some movement, heartbeat is fast (72 hpf)

Wine 2	0	11	0	Both longer than the previous day, yolk sacs are still prevalent, fins and spots evident, shorter tail (48 to 60 hpf); both longer, yolk sacs still attached, quite yellow (60 to 72 hpf)

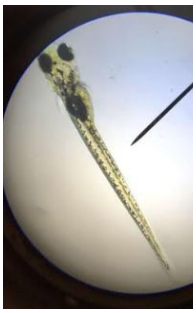
Wine Well 2 *Wine Well 2* *Wine Well 1* *Control Well 1*



Ethanol Well 2 *Ethanol Well 1* *Etanol Well 1* *Control Well 2*






Data Analysis

Table 6. Percentage of Living Zebrafish

Hours Past Fertilization (hpf)	*Control Solution (%)*	*Ethanol Solution (%)*	*Wine Solution (%)*
6	0	0	0
30	0	0	0
54	34.8	52.2	16.7
78	73.9	60.9	75
102	65.2	56.5	70.8

Graph 1. Percentage of Living Zebrafish at Each Time Interval

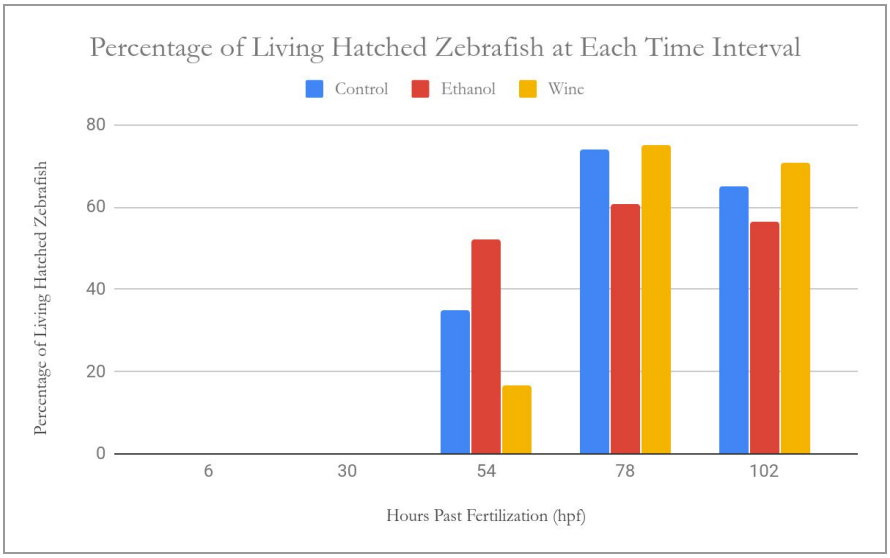


Table 7. Percent Difference of Living Zebrafish at 102 hpf

Solutions Being Compared	*Percent Difference (%)*
Control & Ethanol	14.30
Control & Wine	8.24
Ethanol & Wine	22.47

Table 8. Cumulative Percentage of Dead Zebrafish

Hours Past Fertilization (hpf)	*Control Solution (%)*	*Ethanol Solution (%)*	*Wine Solution (%)*
6	0	0	0
30	8.7	17.4	8.3
54	21.7	26.1	16.7
78	21.7	34.8	16.7
102	30.4	43.5	20.8

Graph 2. Cumulative Percentage of Dead Zebrafish at Each Time Interval

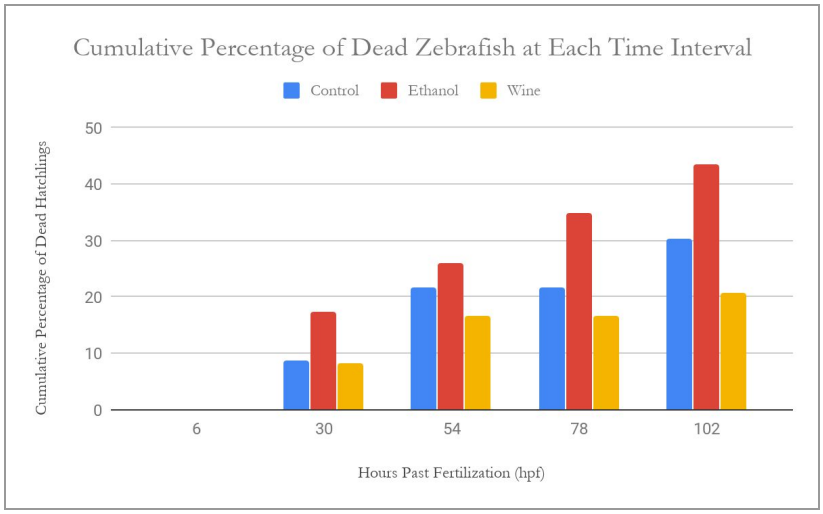


Table 9. Percent Difference of Dead Embryos at 102 hpf

Solutions Being Compared	*Percent Difference (%)*
Control & Ethanol	35.45
Control & Wine	37.50
Ethanol & Wine	70.61

Graph 1 and Table 6 show the percentage of hatched embryos in each type of solution over the 102 hour period. The wine solution had the highest percentage of hatched embryos by the end of the study followed by the control solution and then the ethanol solution. The percent difference was calculated between each solution to see if there was a difference between the extent of hatching. Since the percentages are all greater than 5%, they can all be considered different from each other. Graph 2 and Table 9 show the percentage of dead embryos in each type of solution over the 102 hour time period of the laboratory investigation. The solution with the highest percentage of mortality was the ethanol solution followed by the control solution and then the wine solution. Once again the percent difference between each solution type was

calculated. All of the percent differences were greater than 5%. Therefore, there is a notable difference between the extent of mortality between all three solutions.

In addition to quantitative data, qualitative data was also collected in this experiment through observations. Overall, deformities were noted in every solution type. However, the most notable and numerous deformities were found in the ethanol solution. The observed dead and unhatched embryos were noticeably discolored and deformed unlike any other dead embryos observed in both the wine and control solutions. The ethanol solution also consistently resulted in fish with larger than normal and undissolved yolk sacs, and fish with misshapen tails. The wine solution embryos also resulted in deformities. These deformities consisted of discolored embryo shells and misshapen tails. The control solution embryos started out with just a couple of fish developing flaws, but towards the 102 hours past fertilization, more fish were dying. The cause of these deaths could have been due to the growth of some type of fungus. However, the actual reason for these sudden and unexpected deaths are unknown.

The qualitative data also provided important observations on both the activity and overall development rate of the fish in each solution. The hatched fish in the control solution were quite active. They consistently moved in their wells. The control fish also seemed to be right on track with development. The fish submerged in the ethanol solution were not as active as the control fish and were more sensitive to stimulus. A toothpick was used in order to determine if fish were dead or alive. The fish in the ethanol solution were clearly more affected by this environmental stimulus. In addition, their heart rate and circulation were considerably faster. The development of the fish in the ethanol solution ranged from being behind proper development to being right on track. Lastly, just as the fish in the ethanol solution, the fish submerged in the simulation wine

solution were not as active and had greater sensitivity to stimulus caused by the use of the toothpick. They also seemed to have a faster heart rate and circulation. However, relatively all of the observed fish submerged in the wine solution were not on track with development; they were quite underdeveloped at every observational period.

Discussion

Overall based on the discussion of the collected data, it is seen that the zebrafish that developed in the 100 mM ethanol solution resulted in the lowest percentage of surviving hatchlings, the highest percentage of zebrafish deaths, and the most notable physical developmental flaws. The fish exposed to the simulation wine solution had the highest percentage of surviving hatchlings, the lowest percentage of zebrafish deaths, and some considerable developmental flaws. In addition, it is also important to keep in mind that the control group strayed from expected results due to the possibility that a fungal disease was contracted. Based on the quantitative and qualitative data that was gathered during the duration of this experiment, the hypothesis was not supported. It was originally hypothesized that the simulation wine solution would develop more deformities and have higher mortality due to the fact that this solution contained both ethanol and calcium carbonate. Despite the wine solution having a considerable amount of deformities, the percentage of mortality was low and the percentage of living zebrafish was high compared to the other two solutions that were tested. This low percentage of deaths and high percentage of hatchlings could be due to the sugar that was added to simulate actual wine. The embryos could have potentially used the sugar as an energy source to help aid themselves in development.

All experiments have limitations and errors; thus, this lab is not an exception. Some errors were contaminating living zebrafish, the presence of undissolved particles in the simulation wine solution, and the extended period of time that the embryos were not at their optimal temperature. Contamination of the living embryos was a human error. Nevertheless, it was an error that occurred in this lab investigation. However, the procedure was changed to ensure this error would not carry over into the next days of study. It was very noticeable that the solutes in the wine solution were not completely dissolved all of the time. The solution was stirred before it was pipetted into the wells, but there was no way to keep the mixture completely mixed after it was dispensed. The solution was also quite cloudy. This made observations a bit more difficult. Thus, if this experiment were completed again, it may be more beneficial to use actual wine that has been diluted appropriately. Finally, when the embryos and hatchlings were counted, observed, and given a fresh solution, they were not in their 28.5 °C incubator. Thus, the lower temperature could have lead to unavoidable physical deformities.

The data in this investigation can be linked to a larger body of knowledge. As outlined in the introduction, ethanol is not the only substance that is found in many commonly consumed alcoholic beverages. Alcoholic beverages such as wine have many chemical additives such as potassium sorbate and calcium carbonate. These additives could contribute to developmental defects in zebrafish. Thus, a study such as this one could paint a clearer picture of the role that alcohol has on a developing fetus. After analyzing the collected data, it can not be definitively concluded if wine or ethanol has a more hazardous effect on the physical development of a fetus. There were too many uncontrollable errors in the control group and the other solution types

leading to this inconclusive result. With additional experiments and data, a decisive conclusion may be able to be determined.

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