How Ethanol Causes Premature Births and Deformities in Developing Zebrafish Embryos

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Abstract:

Ethanol creates a serious impact on the health and well being of humans. It causes issues in judgement and mindset, and also physical appearance and internal problems. If an unborn child is exposed to ethanol, it could have some serious birth defects, from unusual appearance to premature births and deaths. To test the effects of ethanol on living creatures, zebrafish were used due to their transparency and easy ability to observe the fish to record data. Forty fish were exposed to different amounts of ethanol, ten embryos were placed in each of the following ethanol concentrations, 0 mM, 30 mM, 100 mM and 300 mM. The results showed that after 48 hours, there was statistical data to support that there was in fact proof to show that zebrafish exposed to ethanol had a premature hatch time. This experiment supported the fact that ethanol has a negative impact on developing creatures.

Introduction:

The primary reason for conducting this experiment was used to demonstrate that alcohol can damage and interfere with the regular development of zebrafish embryos. Alcohol consumption in pregnant women leads to deformities in the child. If a mother consumes alcohol while the baby is still developing, there is a probable chance of brain development issues and problems with developing organs. According to The March of Dimes (2016), there is also a more probable chance of miscarriage, birth defects and premature births. Similar to the fetal development in humans, the exposure to ethanol in the early stages of the zebrafish embryonic development can lead to many abnormalities in the zebrafish. This includes prenatal and postnatal failure to thrive, nervous system deficits, defects in the cardiovascular system, facial structures and limbs. George Streisinger (1981) observed that zebrafish were used as a model organism due to their many advantages. For example, zebrafish embryos are translucent, meaning they are easy to observe everything that is going on externally and internally. The European Bioinformatics Institute (2017) made a statement that zebrafish are very similar to humans by saying, "This latest study shows that the greater part of disease-associated genes in humans have counterparts in the zebrafish." They also have similar body structures as humans such as the backbone and their digestive systems. Plus, the embryos develop very quickly and are very easy to maintain. The

experimental hypothesis was if zebrafish embryos are exposed to large amounts of ethanol then the embryos will have damage that interferes with the development of the embryos, resulting in premature births.

Materials & Methods:

Different treatments of ethanol : (0.0 mM, 30 mM, 100 mM, and 300 mM) (1 ml of each in each well)

- (40) Zebrafish embryos (Ten in each well: Four wells)
- (1) Dissecting microscope (used to observe the fish)
- (4) - 100 mL Beakers (used for dead embryos and unneeded liquid)
- (1) Sharpie
- Instant ocean / embryo media solution (0.0 mM)
- (4) Small bore / pipette (used for sucking up old solutions and transferring new solutions into the well each day of the experiment)
- (4) Large bore / pipette (used for transferring eggs into each well on day 1 and taking out dead eggs the following days.)
- (1) Plate with four wells (container of fish and ethanol)
- (1) 28.5 degrees Celsius incubator (maintains a regulated temperature to increase fish growth)

AS A SAFETY PRECAUTION, GLOVES WERE WORN WHEN HANDLING MATERIALS IN THIS EXPERIMENT

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:

On day one of the experiment, the four wells that will be for containing the fish were set up. The wells were set up going from left to right in order of least amount of ethanol to most amount of ethanol. After the wells were set up, the lid for the wells was labeled with the corresponding amount of ethanol (0 mM, 30 mM, 100 mM, and 300 mM). Using a large pipette, the number of zebrafish used in the experiment was 40 and there was ten zebrafish embryos placed in each well. Any residual liquid from transferring the embryos was removed. After that, each of

the four wells were filled up with 1 mL of each of the assigned concentration of ethanol using a large pipette. Next, the embryos were observed under the dissecting microscope to verify that there were ten embryos in each well. After that, the lid with the labels of ethanol was placed over the wells. Finally, the embryos were placed in an incubator at 28.5 degrees Celsius for 24 hours.

Day 2:

The embryos were taken out of the incubator. The embryos were then examined under the dissecting microscope. All embryos that had turned black were declared deceased and later taken out of the well using a large bore pipette. The live embryos were left in the wells to further develop. After the deceased embryos were removed the old solution was taken out using a small bore pipette. During this part of the lab, care was taken to ensure that no embryos were inadvertently removed while removing the old solution. The old solution was replaced with one mL of the same amount and ethanol concentration from the previous day. The wells were covered up again and placed back into the incubator for 24 hours. Finally, data was recorded and pictures were taken.

Day 3 and 4:

On day three and four, the process of extracting old substances and replacing the old substances with new ethanol was continued as in day two. The embryos were observed under a dissecting microscope and qualitative and quantitative data was recorded. Data included, skin tones, physical appearances, number hatched, alive and dead embryos. and how the fish was deformed. All data and observations were then recorded in table 1.

Day 5:

The experiment was concluded by observing the embryos a final time. Embryos that were still alive and the ones that were dead were recorded and photographed. Final observations were taken including, abnormalities, amount of hatches and behaviors (laying on their side).Chi Square analysis was conducted to determine whether or not the hatch rates were dependent on the concentration of ethanol. The embryos were transferred to a tank filled with other remaining embryos. The remaining ethanol was then extracted from the tray using a fine pipette and then disposed of.

Results:

This experiment was performed to prove the hypothesis that the embryos exposed to ethanol will have development issues and deformities. The dependent variable used in this experiment was the embryos growth and hatch rate and independent variable was the different concentration of ethanol in each well. As the concentration of ethanol increases then the hatch rate will increase. The control used throughout this experiment was the well that contained no ethanol also known as 0 mM. The controlled variables used in this experiment were the same amount of ethanol in each well, same size well, same temperature of ethanol, and same amount of zebrafish eggs used. As the embryos were placed in different levels of concentrations, it was hypothesized that several differences would be documented between the different concentrations. The control well was hypothesized to show no develop issues. Comparing the results of only the control, 0 mM, and the highest concentration of ethanol, 300 mM, there were several differences documented. Differences included, the embryos located in the wells with ethanol (30 mM, 100 mM, and 300 mM) hatched earlier than the embryos in no ethanol (Table 1 and Figure 1). For instance, 48 hours into the experiment, six embryos in the 30 mM, four in the 100 mM and three in the 300 mM had hatched, and began to move. Also in experimental wells, embryos (that had already developed tails and were hatched) had started to develop by getting larger in size and beginning to develope shape for example, they developed straight backs. After 72 hours into the experiment, it was documented that most of the embryos were hatched in all of the wells. Also at 72 hours into the

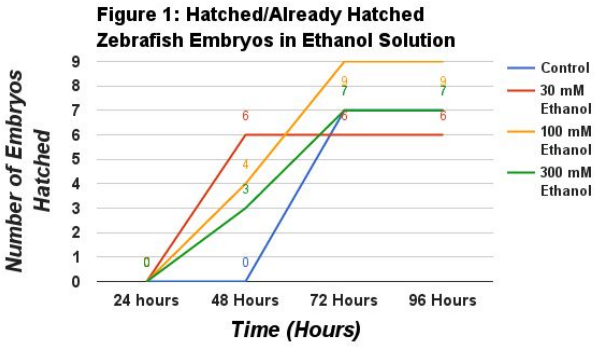

Figure 1 Hatched/Already Hatched Zebrafish Embryos In Ethanol Solution: Display of the number of hatched zebrafish embryos total at the time observed. The most significant piece of data was collected at 48 hours when no embryos were hatched in the control yet all other well had multiple hatches.

Figure 2: The control (0 mM) on day two of the experiment. No embryos were hatched yet. Every other well had three or more that have hatched.

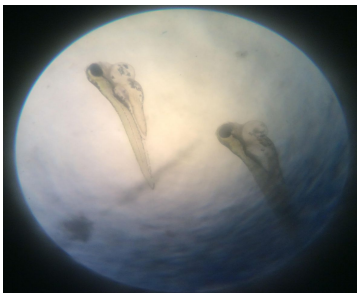

Figure 3: Well 300 mM on day 4 of the experiment. All have hatched but one and are also showing green discoloration.

experiment, black eyes had begun to develop among the embryos located in the control. The only difference with the eyes that was observed was that the experimental embryos had developed their eyes slightly earlier than the control. Both eyes in both experimental and the control was black. In addition, a slight discoloration, greenish skin tone, was observed in the hatched fish of the experimental wells, those containing ethanol at 96 hours into the experiment (Figure 3). It was also noted in the experimental wells, a lot of the zebrafish were on their side (shown in Figure 4), while there were no zebrafish in the control that were on their sides (shown in Figure 5). A pipette was placed right next to the zebrafish, it was recorded that the newly hatched zebrafish in the control was a lot more reactive compared to the experimental wells that were a lot less active meaning they didn't move around much. On day four, one egg remained in the 300 mM well. The embryo didn't appear to be alive and never seemed to hatch therefore was declared deceased (shown in Figure 6). A Chi Square statistical analysis was performed to see the relation between embryos hatched and unhatched in each solution of ethanol. This null hypothesis was accepted with a value of 3.809, critical value of 7.82 and 3 degrees of freedom. Another Chi Square statistical analysis was performed to compare the hatch rate of the different treatments of the zebrafish embryos at 48 hours. The Chi Square value was 10.446. A critical value of 7.82 and 3 degrees of freedom was used to determine significance. This null hypothesis for 48 hours was rejected.

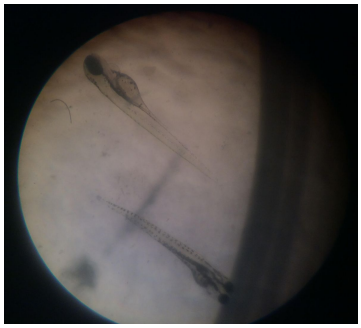

Figure 4: Well 100 mM on day four of the experiment. All but one has hatched at this point in time. They are also on their sides.

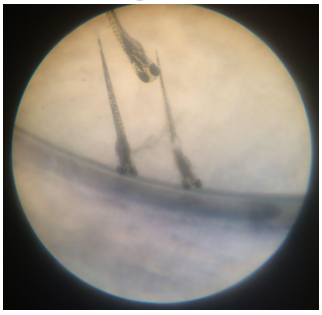

Figure 5: The control (0 mM) on day four of the experiment. By this point all alive embryos had hatched. Also, these fish are all upright instead of on their sides.

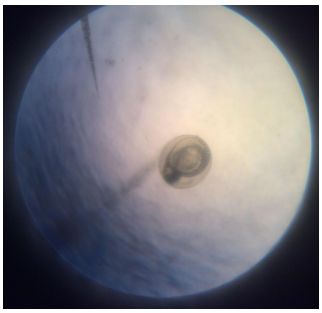

Figure 6: Well 4 (300 mM) on day 4 of the experiment. Above is the egg that was unhatched and declared deceased.

Table 1: Alive and Hatched Embryos In different Concentrations of Ethanol

Treatment	Well #	# of starting fish	24 Hours Post Fertilization Alive	24 Hours Post Fertilization Hatched	48 Hours Post Fertilization Alive	48 Hours Post Fertilization Hatched	72 Hours Post Fertilization Alive	72 Hours Post Fertilization Hatched
0 mM ethanol	1	10	7	0	7	0	7	7
30 mM ethanol	2	10	6	0	6	6	5	5
100 mM ethanol	3	10	9	0	4	4	9	9
300 mM ethanol	4	10	9	0	3	3	8	7

Discussion:

Research was conducted using other reliable resources on the effects of ethanol on humans; this information was then used to further understand the effects of ethanol on zebrafish. It was found that ethanol can cause premature births in humans, as well as brain damage, birth defects, low birth weight, miscarriage and stillbirth; this is because alcohol, when consumed by the mother, travels through the mother's blood and eventually travels into the womb, then to the baby. When the alcohol reaches the baby, the baby then starts to develop problems. There is a similar concept with zebrafish embryos. When the ethanol was placed in the well with the embryos, the ethanol surrounded the embryos right away, in which they then starts to have development issues. This proves that not only does ethanol affect zebrafish embryos but also implies there may be negative effects on the fetal development of humans.

The hypothesis for the experiment was if zebrafish embryos are exposed to large amounts of ethanol then the embryos will have developmental issues that can cause premature births. At 48 hours, all of the wells except for the control had some embryos hatch which proves that the alcohol affected embryos start to develop faster than the control embryos. This also backs up the the research which stated that ethanol can cause premature births in embryos.The experimental data supported the experimental hypothesis. This conclusion was determined by conducting a 48 hour Chi Square Statistical Analysis. This null hypothesis was rejected, which means that their was a correlation between the hatch rate and the treatment. This proves that the data collected was not a coincidence and that the premature births of the embryos was caused by exposure to ethanol.

However, the null hypothesis for Chi Square at the end of five days was accepted and therefore does not correlate with the experimental hypothesis. It was accepted due to fish in the control that started to hatch and catch up to the number of fish in the ethanol solutions. Limitation of the experiment included a short time period for observation, a small number of zebrafish to record, and sources of errors include not taking enough data. For better results, the experiment should have included a larger quantity of zebrafish over a longer period of time, longer observation time with more in depth data collection, and multiple trials. An unanswered question for further research would be whether or not ethanol affects the long term life expectancy of zebrafish. If ethanol does affect zebrafish embryos, then would the surviving fish exposed to ethanol have the same life expectancy as the control fish? Would the effects of ethanol such as cardiovascular, developmental and nervous system issues carry over to their offspring? Further tests are required to understand the full impact of ethanol exposure during early development.

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