The Effect of Artificial Sugar on the Embryonic Development of *Danio rerio*

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Abstract

Aspartame is a chemical sweetener that is used in a variety of food and beverages and is sold under the brand name Equal (Reshman, Sumitha, & Parvathi, 2015). Artificial sugar use is becoming more popular around the world, and there has been debate about the possible effects that artificial sugar could have on the body and on a growing embryo (Setti, Paes De Almeida Ferreira Braga, Halpern, et. al., 2017). Although some studies show that artificial sugar consumption is safe, others show that artificial sugar consumption could potentially be hazardous and could be linked to cancer ("Pros and Cons," 2018). The following experiment aimed to observe if artificial sugar affects the embryonic development of *Danio rerio* (zebrafish). Zebrafish embryos were exposed to sugar and artificial sugar solutions, as well as a solution without any type of sugar, 24 hours post fertilization (hpf). The results show that the phenotypes of the zebrafish in the control and artificial sugar solutions were similar (see Figures 4 & 5), but more zebrafish died when exposed to the artificial sugar solution: 50% of zebrafish in the artificial sugar solution were dead compared to the 21.213% of zebrafish in the control solution that died and the 20.278% of zebrafish in the sugar solution that died at 96 hpf (see Figure 3). Even though this data is not statistically significant, it corroborates with other data to support the idea it is possible that artificial sugar could affect embryonic development. Because of this, an answer to the following question needs to be decided on soon as the popularity of artificial sugar use increases: should women consume artificial sweeteners while pregnant?

Background Information

Zebrafish embryos have several characteristics that make them great model organisms for the study of the effect of various chemicals on vertebrate development, including their rapid development, their transparent eggs, and their ability to be produced in large quantities (Petering, Berg, Tomasiewicz, et. al., 2018). These characteristics allow for not only a large sample size but also for observing the complete development of a zebrafish from a single cell to a complete organism. Furthermore, since zebrafish are vertebrates that have many commonalities with humans, the findings from experiments using zebrafish can often be extended to humans. Zebrafish and humans share about 70% of their genes, which demonstrates that many of the structures, traits, and development pathways have been conserved over time and thus are significant in both organisms ("Why use the Zebrafish," 2014).

Because zebrafish are excellent model organisms for humans, zebrafish were used in the following experiment to test if artificial sugar has an effect on embryonic development. Artificial sugars are widely used in foods such as soft drinks, candy, baked goods, and canned foods, and they are often used to help people with diabetes and help others control their weight ("Pros and Cons," 2018), and because of the "prevalence of overweight and obesity" in society, artificial sweetener consumption has increased dramatically over recent decades (Setti, et. al., 2017). However, even though artificial sweeteners are commonly used, there has been much discussion and debate over the possible health consequences that they have (Setti, et. al., 2017).

One of the main types of artificial sweeteners is aspartame, a chemical that is sold under the brand names Equal or NutraSweet and is 200 times sweeter than sugar ("The Potential Toxicity," 2008). Although studies from health organizations have allowed the FDA to declare

aspartame safe for consumption (“Pros and Cons,” 2018), it has been proposed that aspartame can cause a variety of health problems, including lymphomas, headaches, and dizziness (“The Potential Toxicity,” 2008). In a study at Uva Wellassa University that researched the possible health risks associated with aspartame in zebrafish, it was found that there were observable malformations in zebrafish embryos only when exposed to high concentrations of aspartame (“Toxicity Effects of Aspartame,” 2018). A similar idea is corroborated by the data collected in a study relating preterm delivery with the consumption of artificial sweetened drinks, which shows that there is “a positive association between the intake of artificially sweetened soft drinks and the risk of preterm delivery” but no association for sugar-sweetened drinks (“Intake of Artificially Sweetened,” 2010).

The purpose of this experiment was to determine if artificial sugar (Equal) had an effect on embryonic development in zebrafish. Zebrafish were treated with control, sugar, and artificial sugar solutions and were observed for 4 days. The number of zebrafish hatched and alive were counted after each 24 hour period. This method allowed for numerical comparison of the effect that artificial sugar had on the embryonic development of zebrafish. The hypothesis was as follows: if the zebrafish are exposed to a artificial sugar solution, then the number of zebrafish embryos that are dead will be higher compared to the groups exposed to a sugar solution and a control solution because the chemical aspartame has shown to cause hazardous health issues in both humans and zebrafish (“The Potential Toxicity,” 2008).

The results of this experiment demonstrated that more zebrafish died when exposed to the artificial sugar solution than the number of zebrafish that died when exposed to the control solution or the sugar solution at 96 hpf (see Figure 3). Although this data wasn’t significant, it is

possible with a larger sample size there could be a strong correlation between consumption of artificial sugar and a higher death rate, suggesting that pregnant women should be warned about the potential effects of artificial sugar on the embryo before consumption.

Materials and Methods

The materials used include 122 *Danio rerio* embryos, one well plate, Instant Ocean mix, sugar, Equal artificial sweetener, five large mouth pipettes, five small mouth pipettes, two glass containers (for storing sugar solutions), one graduated cylinder, a scale, an incubator, and a microscope.

Before the experiment began, the proper solutions of sugar and artificial sugar were obtained. First, 100 mL of Instant Ocean mix was measured in a graduated cylinder while 0.1 g of sugar was measured on a scale. Then, the sugar was poured in the graduated cylinder, and after, the solution was poured into a labeled glass container. This same method was repeated with the artificial sweetener. After the solutions had been prepared, zebrafish embryos were gathered 4 hours postfertilization (hpf) and between 9-12 alive embryos were placed in each well. Each well was then filled halfway with Instant Ocean mix using the small mouth pipette, and the well plate was put in an incubator at 28.5 degrees Celsius. At 24 hpf, the dead embryos were removed and replaced using a large mouth pipette, and the Instant Ocean solution was removed from each well. Then, the well plates were labeled horizontally A, B, or C (each row having 4 wells), with row A receiving the Instant Ocean, row B receiving the 0.001 g/mL sugar solution, and row C receiving the 0.001 g/mL artificial sugar solution. Each well was filled about half way with its respective solution. The embryos were then placed in an incubator set at 28.5

degrees Celsius for about 24 hours. At 48, 72, and 96 hpf, the dead embryos were removed from each well and the solutions of the same concentration as the day before were replaced. The embryos were observed under a microscope for possible deformities and to make sure each embryo removed was actually dead. Then, the number of embryos alive and hatched were recorded, and the embryos were returned to the incubator. By counting the embryos at each 24 hour interval, a numerical and statistical comparison between each solution could be made. At 97 hpf, the embryos and hatched fish were removed from the wells and disposed of in an ethically humane way. The method was based on the procedure followed by the University of Wisconsin - Milwaukee in the module Zebrafish as Models (Petering, et. al., 2018).

Several safety precautions were observed throughout the experiment. First, no one in the lab touched or came into direct contact with the embryos; large mouth pipettes were used for all transport of the embryos. Additionally, no one ingested any of the solutions that were used on the embryos. Lastly, all materials were carefully handled and placed in the center of the lab bench to prevent anything from breaking or getting mixed together.

Results

In this experiment, the independent variable is the type of sugar solution, either sugar or artificial sugar, and the dependent variable is the amount of zebrafish alive. The controls of this experiment were incubation temperature, type of fish embryos, and concentration of the sugar solutions. 9-12 zebrafish embryos were randomly placed into each well, and each row of wells was exposed to a different solution. The number of zebrafish that were alive in each of the three solutions at 24 hpf, 48 hpf, 72 hpf, and 96 hpf were recorded. By counting the number of

zebrafish alive, it will be discerned if the type of sugar solution affects embryonic development. The zebrafish exposed to the 0.001 g/mL of artificial sugar solution had the most zebrafish dead at 24 hpf, 48 hpf, 72 hpf, and 96 hpf. The zebrafish exposed to the control solution had the least amount of zebrafish dead at all time intervals where data was collected, while the zebrafish exposed to the 0.001 g/mL solution of sugar had a lower number zebrafish dead than the control, but the number of zebrafish dead was not significantly different (See Figure 1&2). Overall, 21.213% of the zebrafish exposed to the control solution were dead after 96 hpf, 20.278% of the zebrafish exposed to the sugar solution were dead after 96 hfp, and 50% of the zebrafish exposed to the artificial sugar solution were dead after 96 hfp (See Figure 3).

Figure 1: Data Table of the Effect of Sugar Solutions on the Number of Embryos Alive

The Effect of Sugar Solutions on the Number of Embryos Alive

	24 hpf	48 hpf	72 hpf	96 hpf
Control Solution	43	35	34	34
0.001 g/mL Sugar Solution	39	34	32	31
0.001 g/mL Artificial Sugar Solution	40	29	29	20

Figure 1 shows the number of embryos that were alive at 24 hpf, 48 hpf, 72 hpf, and 96 hpf. At 96 hpf, the most amount of embryos died when exposed to the artificial sugar solution and the least amount of embryos died when exposed to a control solution.

Figure 2: Graph of the Effect of the Type of Sugar Solution on the Percent of Zebrafish Alive

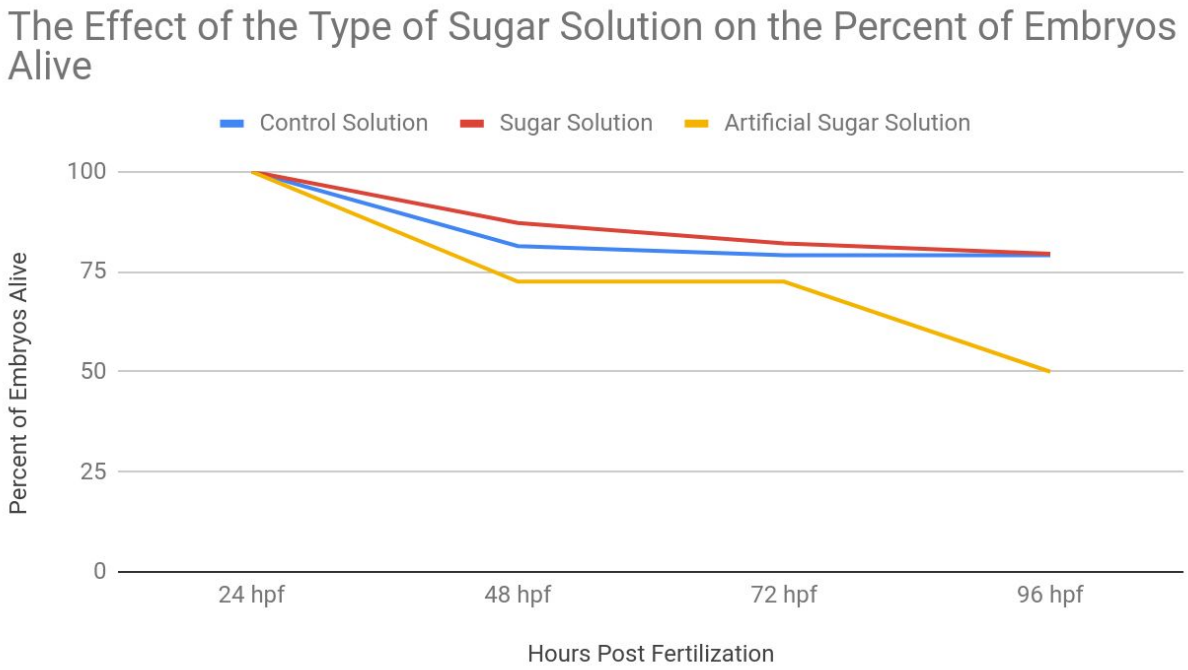

Figure 2 shows the percent of zebrafish that were alive in each of the solutions from 24 hpf to 96 hpf. The embryos in the sugar solution had the greatest percent of zebrafish alive throughout the whole time interval of 24 hpf to 96 hpf while the embryos exposed to the artificial sugar solution had the least percent of embryos alive from 48 hpf to 96 hpf. The percent of zebrafish alive in the control solution was less than the percent of zebrafish alive in the sugar solution, but it was not significantly different. The most rapid decline of the percent of zebrafish alive in all three of the solutions occurred between 24 hpf and 48 hpf, and in the control and sugar solutions, the decline in the number of zebrafish alive was much smaller between 48 hpf and 96 hpf. In the artificial sugar solution, the number of zebrafish alive remained the same between 48 hpf and 72 hpf, but there was a sharp decrease in the number of zebrafish alive from

72 hpf to 96 hpf. At 96 hpf, 79.07% of zebrafish in the control solution were alive, 79.49% of zebrafish in the sugar solution were alive, which isn't significantly different from the control group, and 50% of the zebrafish in the artificial sugar solution were alive.

Figure 3: Graph of the Effect of the Type of Sugar Solution on the Average Percent of Zebrafish Dead

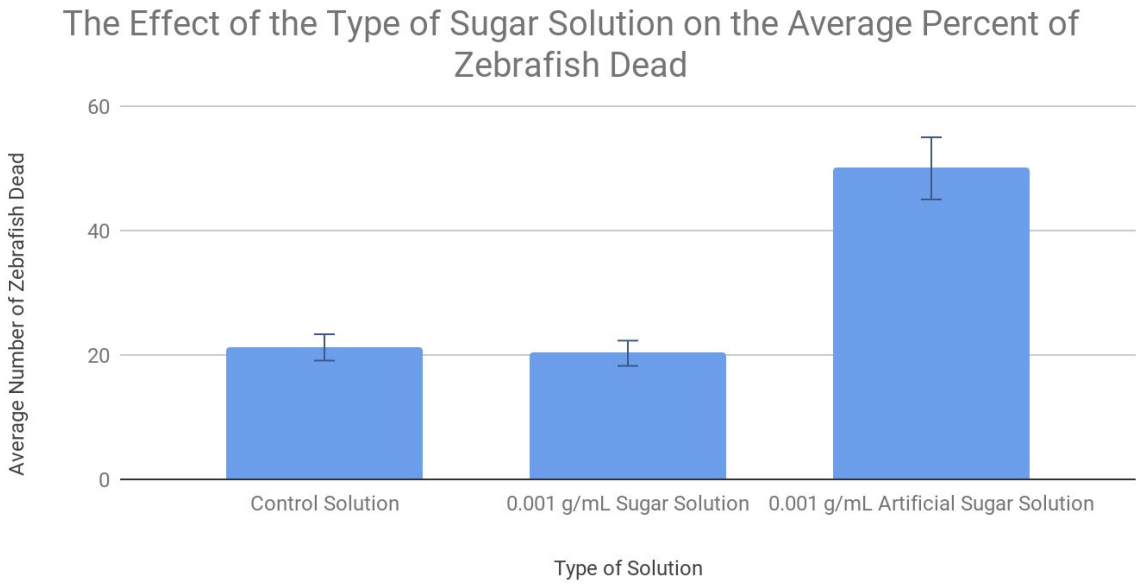

Figure 3 shows the average percent of zebrafish that were dead from 24 hpf to 96 hpf. At 96 hpf, 21.21% of the zebrafish had died when exposed to the control solution, 20.28% of the zebrafish had died when exposed to the sugar solution, and 50.00% of the zebrafish had died when exposed to the artificial sugar solution. Using a t-test, the percent of zebrafish dead in the sugar solution did not show statistically significant data (20.28% ± 5.62, p=0.9170), nor did the percent of zebrafish dead in the artificial sugar solution (50.00% ± 23.45, p=0.2816).

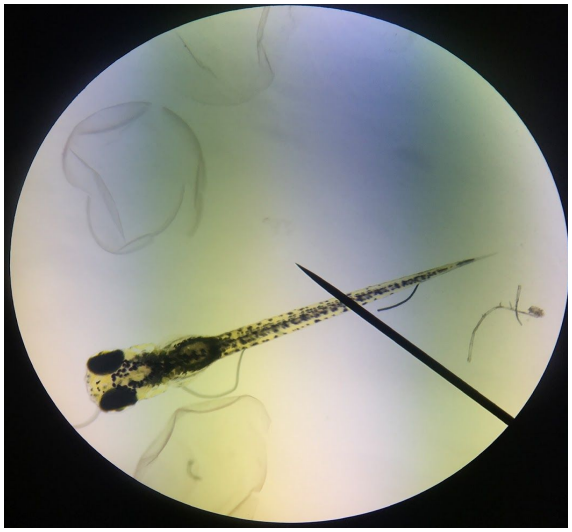

Figure 4: Image of Zebrafish Exposed to Control Solution at 96 hpf

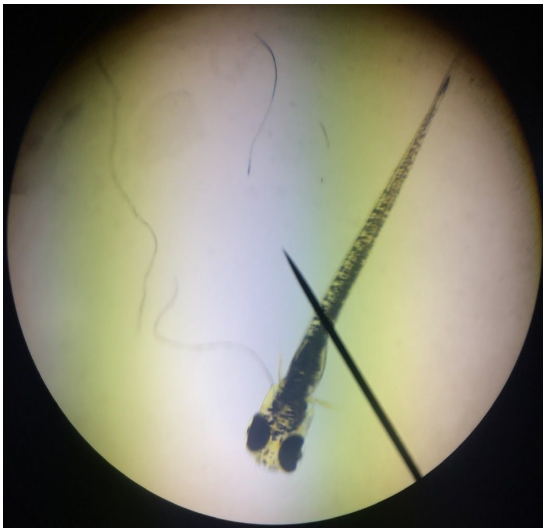

Figure 5: Image of Zebrafish Exposed to Artificial Sugar Solution at 96 hpf

Figures 4 and 5 show a microscopic image of zebrafish exposed to the control solution and the artificial sugar solution at 96 hpf. Both zebrafish have dark pigment from their head down their tails, and they are also about the same size. These images reveal that the zebrafish appear to have developed similarly and that there aren't any apparent differences between the organisms' structures and phenotypes.

Discussion

Overall, the zebrafish exposed to the artificial sugar solution had a higher percentage of zebrafish dead than both the zebrafish exposed to the sugar solution and the zebrafish exposed to the control solution from 24 hpf to 96 hpf. However, the phenotypes of the zebrafish exposed to the artificial sugar solution and control solution were very similar. The zebrafish in the sugar

solution had the lowest percentage of zebrafish dead while the control solution had a similar percentage of zebrafish dead to the sugar solution.

The results of this experiment show that artificial sugar does not significantly affect embryonic development. The hypothesis written before this experiment is as follows:

Hypothesis 1) If the zebrafish are exposed to a artificial sugar solution, then the number of zebrafish embryos that are dead will be higher compared to the groups exposed to a sugar solution and a control solution because the chemical aspartame has shown to cause hazardous health issues in both humans and zebrafish ("The Potential Toxicity," 2008).

Hypothesis 1 is not supported by the data from this experiment because the data doesn't prove a correlation between artificial sugar and a higher death rate. Since the p value comparing the control and artificial sugar groups is 0.2816, the data is not significant, and therefore no correlation can be proved. This is most likely because of the high standard of error for the artificial sugar experimental group (23.452%). However, when the data for the average percent of zebrafish dead in the control and artificial sugar solutions is repeated three times for statistical analysis purposes, the p value comparing the two equals 0.0337, which means that that data is significant. This suggests that a larger sample size could mean statistically significant data, and thus a correlation between exposure to artificial sugar and a higher death rate.

In an experiment performed at Sri Ramachandra University, zebrafish embryos were exposed to higher concentrations of the chemical aspartame, which is the primary chemical in Equal artificial sugar (Reshman, et. al., 2015). At concentrations of 125 mg/mL, 250 mg/mL, and 500 mg/mL, 100% of the zebrafish were dead after 48 hours (Reshman, et. al., 2015). The zebrafish in that experiment were exposed to a much higher concentration of aspartame than this

experiment, which suggests that higher doses of aspartame can be much more fatal than lower doses. The results of the experiment above show a much more significant correlation between high levels of aspartame and a higher death rate than the experiment discussed in this paper, but it can still be inferred that artificial sugar has some impact on embryonic development, which is why pregnant women should not consume it. Furthermore, it is possible that pregnant women are consuming much more than the approved dosage of aspartame (Equal) because it is found in more than 6,000 products and is common in today's diet, so many pregnant women could be at a higher risk of having a miscarriage. (Setti, et. al., 2017). For this reason, if this experiment were to be repeated, concentrations similar to and larger than the approved dosage of aspartame, 40 mg/kg/d, would be used in order to create a stronger connection to human health (Setti, et. al., 2017). Similarly, in another study of over 59,000 pregnant women, a positive association was seen between preterm delivery and artificial sugar consumption, but there was no association between preterm delivery and sugar consumption ("Intake of Artificially Sweetened," 2010). The evidence provides suggests that artificial sugar does impact the human embryo in utero whereas regular sugar does not. This evidence is corroborated in this experiment with zebrafish because the embryos in the sugar solution had a similar death rate to those in the control solution but many more embryos died in the artificial sugar solution, showing that they type of sugar solution possibly had some effect on embryonic development. Even though the data didn't prove to be significant with the sample size used, a larger sample size could potentially show a greater effect of artificial sugar on the embryonic development of zebrafish, so if this experiment were to be repeated, a larger sample size would be used.

The standard error for the zebrafish exposed to the artificial sugar was 23.452%, indicating that there were significant errors that occured in this experiment. First, when the artificial sugar was put in the petri dish to be measured, some of it was stuck to the bottom of the petri dish, which meant that the correct amount of artificial sugar may not have been put in the solution. This could have impacted the results because the solution may have been less of a concentration that it should have been. Furthermore, there is a possibility of human error in this experiment. It is possible that someone in the lab may have accidentally picked up an alive embryo and removed it, thinking it was dead, or counted a dead embryo as being alive, which would have affected the overall totals and thus the results. Lastly, one limitation to this experiment is the small sample size. There was a lot of variation among the wells exposed to similar solutions, so there were some possibly outliers when the data was averaged. With a larger sample size, a more accurate average would be able to be calculated, which would determine if artificial sugar does have an effect on embryonic development.

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