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

Mya Colón and Lauren Simmons, November-December, 2018

Abstract

This lab was designed to test the teratogenic effects of artificial sweetener, specifically saccharin and sucralose, on *Danio rerio* (zebrafish) embryos by measuring the number of zebrafish that were alive and that hatched every day over the course of 96 hours post fertilization (hpf). In an experiment conducted by Griglak (2016), zebrafish embryos were exposed to a solution of Equal artificial sweetener whose main ingredients are dextrose, maltodextrin, aspartame, and acesulfame potassium. The findings of their experiment suggest that an embryo, if placed in the artificial sweetener solution, had a 30% higher chance of dying than if it were placed in the regular sugar solutions or the control group (Griglak, 2016). This posed the question of how other types of artificial sweeteners and the concentration at which the embryos are exposed would affect the growth and development of the zebrafish. This experiment began with ten zebrafish embryos placed in each well of a twelve-well falcon dish. At 24 hpf, four of the wells were exposed to a 0.0002 g/mL saccharin solution. Simultaneously, four wells were exposed to a sucralose solution containing 0.0002 g of Splenda per mL of Instant Ocean. The last four wells were filled with pure Instant Ocean solution and dubbed the control group. It was anticipated that zebrafish exposed to either of the artificial sweetener solutions would have a higher death rate than those not exposed. After the 96 hpf testing period, the data collected showed that that prediction held true. However, the majority of deaths occurred in the saccharin-exposed group.

Background

Zebrafish As a Model

In this research lab, the goal was to investigate the effect of the chemical compounds saccharin and sucralose on human embryonic development using zebrafish embryos as a model. These embryos have transparent eggs and there is research showing the similarities of the zebrafish genome to that of humans. Additionally, their development cycle is fast-paced, and are relatively low maintenance, which is ideal when using them as test subjects. Finally, they are inexpensive, which allows for purchasing them in bulk. For these reasons, zebrafish serve as a practical model to use when making qualitative observations to investigate the teratogenic effects of artificial sweetener, as is the goal of this experiment (Hill, Teraoka, Heideman, et al., 2005, p. 6).

Substances

Saccharin is sold under the brand name Sweet N' Low, and sucralose is sold under the brand name Splenda. Both have been legalized by the U.S. Food and Drug Association (FDA) as a "non-nutritive sweetener… under certain conditions of use" (U.S. Food and Drug Association, 2018). Despite being approved by the FDA, there have been studies that show reason to stay conscious about potential health issues that may arise because of them. Studies have shown an increase in obesity levels as the use of artificial sweetener has gone up over the past four decades. Additionally, a drastic increase in the chance of developing Type 2 Diabetes has been observed and their risk of developing metabolic syndrome has doubled (Swithers, 2013, p. 431-441). Furthermore, according to the American Pregnancy Association, "saccharin crosses the placenta and may remain in fetal tissue." Therefore, its effects on embryonic development

may be long-lasting and detrimental (2015). The purpose of the lab is to continue presenting knowledge concerning the effects of artificial sweetener on humans, specifically during embryonic development, aiming to collect data that agrees with one or both of the hypotheses this lab aimed to prove.

Hypotheses

- If zebrafish embryos are exposed to saccharin and sucralose, then the hatched fry will show proof of internal dysfunction and phenotypic abnormalities by the end of the 96 hpf experimental period because artificial sweeteners like saccharin and sucralose have been observed to have negative effects on the human body.
- If zebrafish embryos are exposed to saccharin, then the hatched fry in this experimental group will show the most severe signs of mutation out of all of the control and experimental zebrafish.

The research of Kim, Seo, and Cho (2011) confirms the hypothesis that saccharin results in physical mutations, shown by observing swimming defects in their experimental zebrafish, as well as internal mutations such as increased cholesterol and serum glucose levels. High blood cholesterol levels in adult humans have been found to result in "the buildup of plaque deposits in blood vessels throughout the body," which, if left untreated, can result in carotid artery disease, coronary heart disease (including, but not limited to, angina or heart attack), peripheral artery disease, and stroke (National Heart Blood and Lung Institute, 2014). Additionally, it has been found that hyperglycemia (high blood glucose levels) can result, in addition to microvascular disease and other macrovascular complications, in oxidative stress in the patient (Marfella, Quagliaro, Nappo, et al., 2001, p. 635-636). According to this research team, oxidative stress can

be classified as a "common downstream mechanism by means of which multiple byproducts of glucose are exerting their adverse effects on blood vessels." If saccharin, as investigated by Kim, Seo, and Cho (2011), affects the internal development of zebrafish by increasing cholesterol and serum glucose levels, it is inferred that fully-developed, adult zebrafish may experience some type of heart or vascular disease, as well as oxidative stress. Similarly, in a study using rats it was found that sucralose can greatly reduce the amount of beneficial microbial life inside the gastrointestinal tract (Schiffman & Rother, 2013, p. 399-451). The microbial life found within the intestines serve an extremely integral role in digestion, the production of essential vitamins, and protection of the gastrointestinal tract from pathogen colonization" (Hillman, Lu, Yao, et al., 2017). These findings indicate that any interference with the microbial colonies in the gastrointestinal tract may lead to the system's inability to function correctly. Therefore, if sucralose, as investigated by Schiffman and Rother, interferes with the amount of microbial life within the gastrointestinal tract, then it can be inferred that fully-developed, adult zebrafish may experience some sort of digestive system failure later in life. Due to the results of the experiment and others like it, it's important that further research be performed to benefit society's understanding of the effects artificial sweeteners can have on one's body. This is an urgent matter due to the rising amounts of foods containing artificial sweeteners.

Materials & Methods

The variable being measured in this experiment was the variation in physical effects on the embryonic development of zebrafish when exposed to either saccharin or sucralose. Data was then measured over the course of time, 24 hpf to 96 hpf scaled by 24 hours. Throughout this time period, the type and concentration of solution, breed of fish, time since fertilization, room and incubator temperature, and beginning amount of embryos in each well remained constant. One hundred and twenty zebrafish embryos were used, forty of which were controlled, and eighty of which were experimental (forty per each experimental group). Row A contained the control solution, which was a diluted Instant Ocean solution. Row B contained the saccharin experimental group, the solution being concentrated at 0.0002 grams of saccharin per 1 mL of Instant Ocean. Row C contained the sucralose experimental group, the solution being concentrated at 0.0002 of sucralose per 1 mL of Instant Ocean.

Observations were made at 24 hpf, 48 hpf, 72 hpf, and 96 hpf. Notes were taken on specific wells, recording phenotypic abnormalities such as curvature of the spine and reaction time. The number of live fish and the number of hatched fish were recorded. At 96 hpf, the final amount of living and hatched eggs were recorded, and the eggs and fish that were remaining were disposed of by method of freezing.

The materials used in this experiment were Instant Ocean solution, Sweet'N Low artificial sweetener (saccharin), Splenda artificial sweetener (sucralose), zebrafish embryos, 100 mL bottles to hold the saccharin and sucralose solutions, a graduated cylinder and stirring rod, a twelve-well falcon dish, wide-mouthed and fine-tipped transfer pipettes, a bi-optic microscope, and an incubator for storing the zebrafish embryos. During this experiment it was made sure that

proper safety precautions were taken such as wearing closed-toed shoes in lab, tying back long hair and loose clothing, and washing hands after handling the solutions and embryos.

Procedure

1. Place ten zebrafish embryos into each well of the twelve-well falcon dish with a pipette.
2. Remove all of the dead and unfertilized eggs and dispose in waste using a pipette.
3. Remove liquid from all of the wells and dispose in waste beaker. Replace liquid with fresh Instant Ocean solution making sure to fill each well half way.
4. Place falcon dish with embryos into the incubator.
5. Prepare saccharin (Sweet'N Low) and sucralose (Splenda) solutions (0.0002 gm/mL).
    a. Measure 100 mL of Instant Ocean solution.
    b. Measure 0.02 gm of saccharin.
    c. Mix these values of Instant Ocean and saccharin.
    d. Place the saccharin solution in a bottle and label it.
    e. Repeat steps (a) through (d) with sucralose.
6. At 24 hours post fertilization, take falcon dish out of incubator and remove any dead embryos and old solution from all wells using a pipette.
    a. Refill the first four wells halfway with fresh Instant Ocean solution and label it.
    b. Expose the next four wells to saccharin solution. Label the row and fill halfway up.
    c. Expose the last four wells to sucralose solution. Label the row and fill halfway up.
    d. Record how many fish are alive and how many have hatched in each well.

    e. Put the lid on the falcon dish and store in the incubator.

7. At 48 hours post fertilization, take out the falcon dish.
    a. Remove dead embryos and dirty solution from each well using a pipette.
    b. Refill each well with the correct solution (Instant Ocean, saccharin, or sucralose solution).
    c. Record the number of living and hatched zebrafish in each well.
    d. Take note of any phenotypic abnormalities in the zebrafish fry.
    e. Close the falcon dish and place in the incubator.
8. Repeat all parts of step seven at 72 and 96 hours post fertilization.
9. Once all data collection is complete, remove all of the fish and eggs from the falcon dish using a wide-tipped syringe and place them in a petri dish for disposal.
10. Clean out the falcon dish making sure to follow proper lab safety guidelines.

(Petering, Berg, Tomasiewicz, et al., 2014, 14-25)

Results

This lab tested the manner in which artificial sweetener, specifically saccharin and sucralose, affected the mortality and hatch rate of zebrafish embryos.

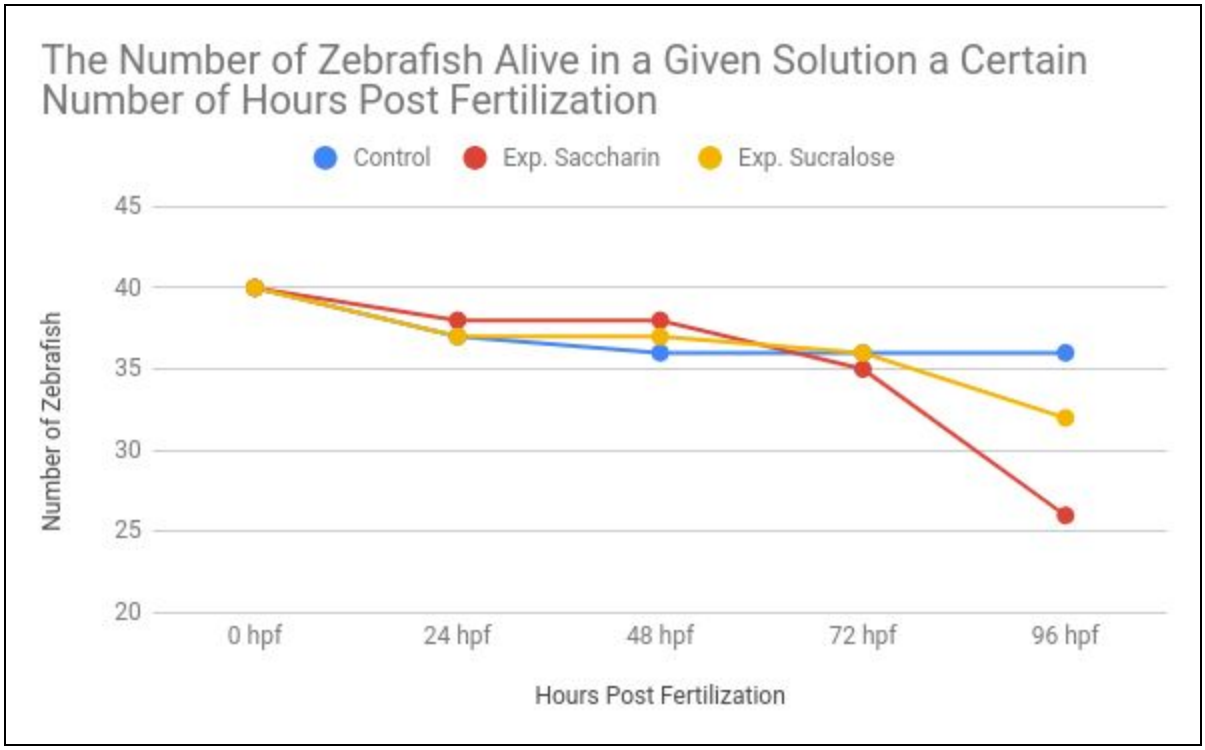

Figure 1

The number of zebrafish alive after 96 hpf was highest in the control group compared to both the saccharin and sucralose experimental groups. However, there were fewer saccharin-exposed zebrafish alive than those exposed to sucralose post 96 hpf. As seen in *Figure 1*, all three groups began with the same number of zebrafish (ten per well). At 24 hpf, each group experienced a decrease in amount of living zebrafish, the more significant decrease happening in the control and sucralose experimental groups. At 48 hpf, only the control group dropped a small number due to an individual death. At 72 hpf, the zebrafish in the saccharin and sucralose groups

decreased while the control group remained the same. In both experimental groups, there was a significant decrease in living zebrafish fry by 96 hpf, with the saccharin group having the most drastic change with a count of nine dead fish over the course of 24 hours.

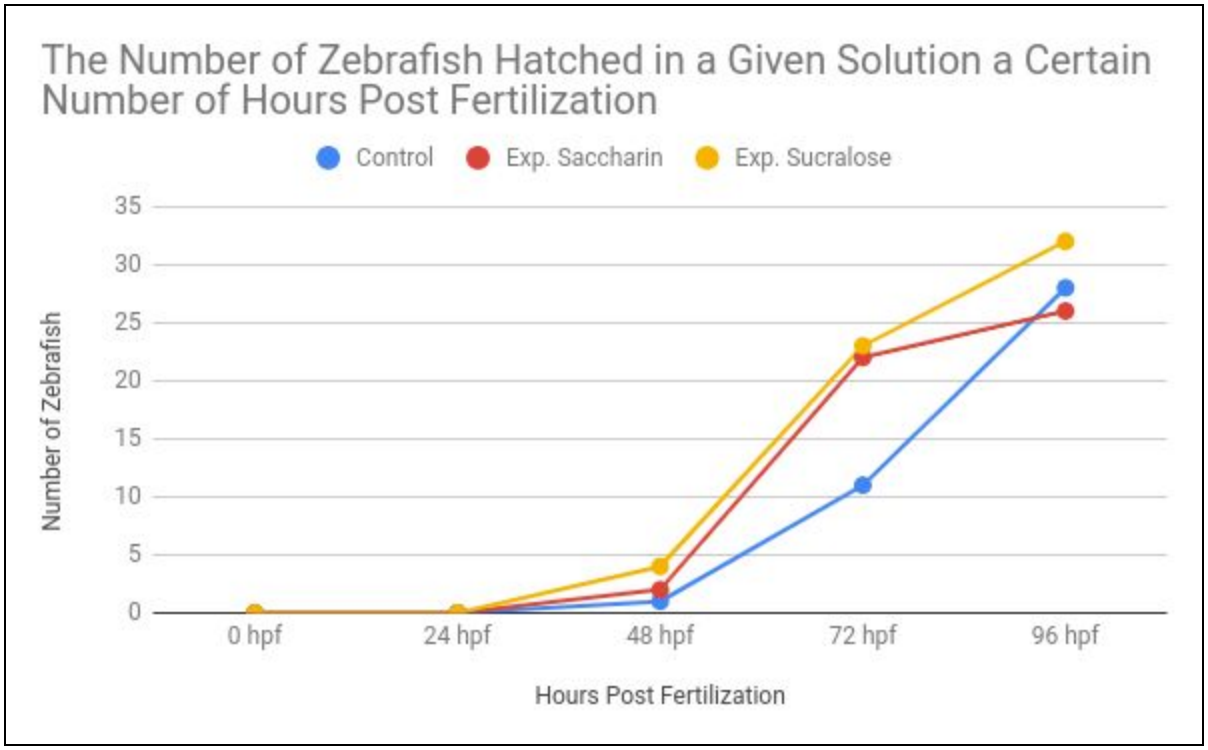

Figure 2

By the end of the 96 hpf period in which observations were made, the amount of live zebrafish hatched varied greatly between the three groups being compared with the saccharin experimental group having the fewest hatched fry and the sucralose experimental group having the most hatched fry. By looking at *Figure 2*, it can be discerned that the zebrafish did not start hatching until 48 hpf. At this time, the most zebrafish hatched were in the sucralose group, followed by the saccharin experimental group, and finally the control group with the fewest fry

hatched. At 72 hpf, there was a significant increase in hatched zebrafish in all three groups. The more drastic increases in hatched fry were in the two experimental groups. There was a great increase in the number of zebrafish hatched in the control group at 96 hpf.

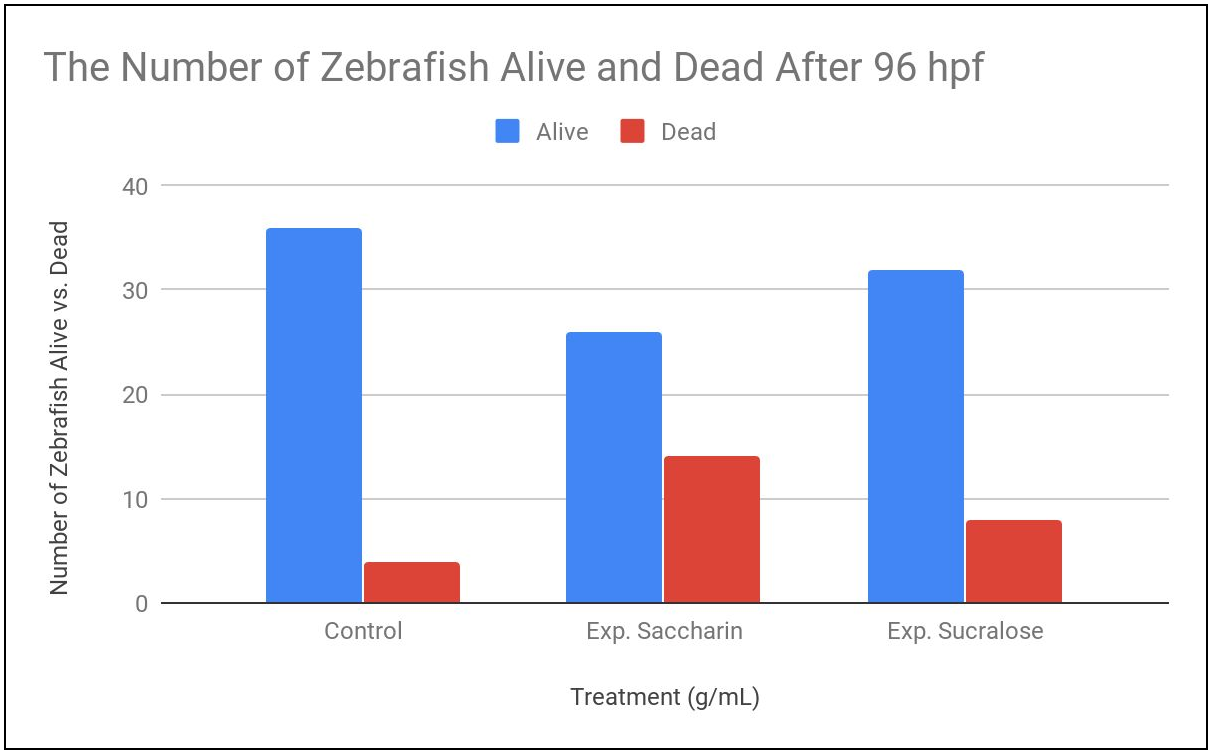

Figure 3

Figure 3 represents the disparities between the three groups in the number of zebrafish that were alive and dead by the end of the 96 hour experimentation period. Although more zebrafish died when placed in the sucralose solution rather than the Instant Ocean (control), it's evident by looking at the graph that embryos placed in the saccharin solution were more likely to die within this time period compared to those placed both in the sucralose and control solutions.

Statistical Significance

The statistical test used to show the difference between the controls and experimental groups being measured was the unpaired *t*-test, which is made for comparing the means of two groups of independent data (Oxbridge Solutions Ltd., 2013). In this experiment, these independent groups were the zebrafish eggs hatched in the Instant Ocean solution (control), and the zebrafish eggs that were hatched in the experimental solutions (saccharin and sucralose).

	p-value*
Alive 96 hpf, saccharin	0.2622
Alive 96 hpf, sucralose	0.3153
Hatched per day, saccharin	0.8128
Hatched par day, sucralose	0.3153

The data is not considered significant according to the p-values above. However, the data could have been statistically significant if there was a larger sample size. By replicating the raw data from this experiment four times and calculating the significance, it was determined that the p-values for the amount of remaining live zebrafish after 96 hpf would have equaled 0.0096 (saccharin) and 0.0204 (sucralose), and for the amount of hatched zebrafish per day equaled 0.5842 (saccharin) and 0.0204 (sucralose) if there was a larger sample size.

*These values were determined using a scale where $p \leq 0.05$ is significant (GraphPad Software, Inc. 2016).

Qualitative Images

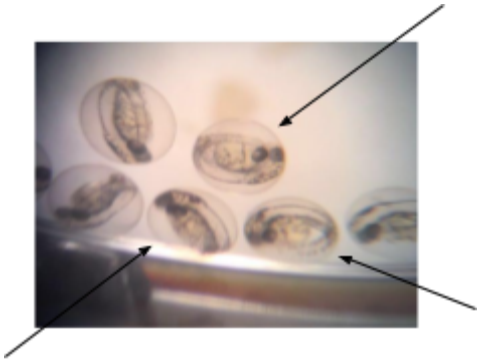

Illustration 1: Zebrafish Embryos in Control Group 48 hpf.

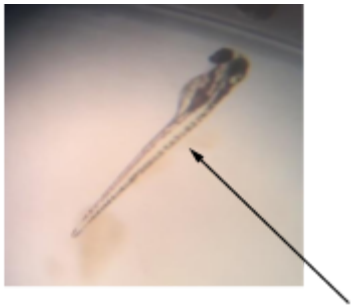

Illustration 2: Zebrafish Fry in Control Group 96 hpf.

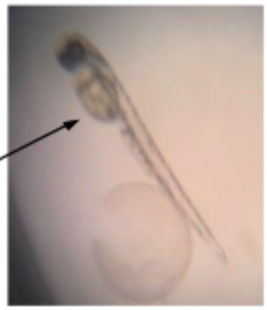

Illustration 3: Zebrafish Fry in Saccharin Solution 48 hpf.

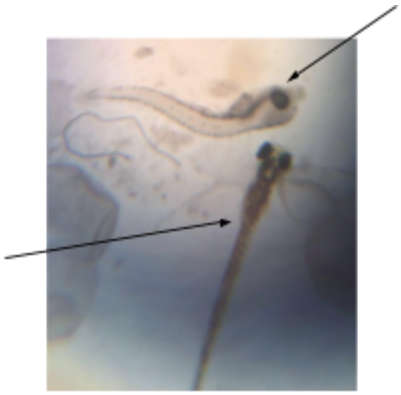

Illustration 4: Dead (Top) and Living (Bottom) Zebrafish Fry in Saccharin Solution 96 hpf.

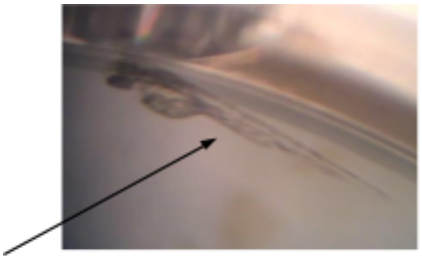

Illustration 5: Zebrafish Fry in Sucralose Solution 48 hpf.

Illustration 6: Zebrafish Fry with a Bent Spine in Sucralose Solution 72 hpf.

Illustration 7: Zebrafish Fry in Sucralose Solution 96 hpf.

Observations and Sources of Error

The first substantial observations made during this experiment were at 48 hpf when fry started hatching. In the sucralose experimental group, four zebrafish fry were hatched, which is extremely early when compared to the control group that only had one fish hatched by 48 hpf. This trend of early hatching was proven constant in both experimental groups as the time frame continued on to 72 hpf and 96 hpf and both saccharin and sucralose experienced a major increase in the amounts of hatched zebrafish fry in comparison to the hatch rate of the control group. These observations are supported by the data found in *Figure 2*.

The first records of phenotypic abnormalities were taken at 72 hpf. The earliest and most apparent abnormality noticed were the lighter markings along the backs of the zebrafish fry in the saccharin experimental group (*Illustrations 3 and 4)*. While the control group fry had translucent bodies covered with dark spots (*Illustration 2*), the zebrafish exposed to saccharin had significantly fewer dark marks. As time continued on to 96 hpf, this abnormal phenotype was noticed to be apparent on all fish with curved spines (*Illustrations 4 and 6*), which was notable only a prevalent issue in the saccharin experimental group.

At 96 hpf, after noticing at the sucralose experimental group had experienced rapid hatching rates in comparison with the controlled group, it was also recorded that the reaction times of these fry were extremely delayed. When gently poked with a fine-tipped pipette, the sucralose-exposed fish had to be prodded two to four times in order for them to register the disturbance and swim away, whereas the controlled fish would dart away at the slightest tap of the pipette.

One error that may have affected the reliability of our data is as follows: At 72 hpf, while recording the number of living and hatched zebrafish, it was noticed that in two fish were missing from well B2 and one missing from B3 (saccharin-exposed experimental), as well as one missing from C2 (sucralose-exposed experimental). No errors of this kind occurred within the control group. This error most likely occurred via a pipetting mistake while disposing of waste and accidentally removing a zebrafish fry.

Discussion

The purpose of this lab was to collect data that agrees with one or both of the hypotheses this lab aimed to support:

- If zebrafish embryos are exposed to saccharin and sucralose, then the hatched fry will show proof of internal dysfunction and phenotypic abnormalities by the end of the 96 hpf experimental period because artificial sweeteners like saccharin and sucralose have been observed to have negative effects on the human body.
- If zebrafish embryos are exposed to saccharin, then the hatched fry in this experimental group will show the most severe signs of mutation out of all of the control and experimental zebrafish.

The qualitative data collected from this lab partly supported hypothesis 1 by showing how both experimental groups had curved spines, lighter markings, and delayed reaction times. However, the majority of mutated and dead zebrafish fry by the end of the 96 hpf period were in the saccharin-exposed experimental group. This more accurately represents hypothesis 2 as it predicted that the saccharin-exposed zebrafish would experience long-lasting detrimental effects from these mutations. The saccharin experimental group had an overwhelming amount of curved spine, light-colored fry, and were responsible for most of the death count at the end of the 96 hpf lab period (*Figure 3*). It can be inferred that the effect of saccharin on the embryonic development of zebrafish was the most significant due to the low p-value (0.0096) that was associated with the amount of dead zebrafish (*Figures 1 and 3*) between the control group and the saccharin-exposed experimental group. This indicates the need for further research with its effects during the embryonic development stages as well as its long-lasting effects on the adult

life of the zebrafish. Both of these tests would use a much larger sample size. This would ultimately increase the understanding of how this specific artificial sweetener may negatively affect zebrafish in the developmental stages and later in life.

Although the data acknowledges all of the predictions made in the original two hypotheses that were focused on during this lab to an extent, the data collected in *Figure 2* calls for further investigation. As the models used in this experiment were zebrafish, and one of the reasons why they were used was because their genome is similar to that of a human's, it is important to explore the possibility of the data collected from this lab could also apply to humans. In *Figure 2,* the data shows that the sucralose-exposed group had a significantly larger amount of hatched fish by the 48 hpf checkpoint compared to the other two groups; this could suggest that exposure to sucralose during embryonic development may result in premature births in humans. Currently, there is little to no research on how sucralose may affect the organism's chances of having a premature birth. By conducting further tests regarding the effect of sucralose on the hatching rate of zebrafish embryos there will be further evidence to support the idea that sucralose, while ingested during the gestation period of a female human, may result in an increased risk of premature birth, which may lead to complications later in life.

While the inferences that saccharin can increase the risk of early-stage death and phenotypic abnormalities are backed by evidence shown in *Table 1* and *Figures 1-3,* it is important to recognize the potential for error in this lab. One error that may have affected the reliability of our data is as follows: At 72 hpf it was noticed that two fish were missing from well B2 and one missing from B3 (saccharin-exposed experimental), as well as one missing from C2 (sucralose-exposed experimental). This error most likely occurred via a pipetting mistake while

disposing of waste solution. Another important factor to consider when analyzing the data collected is the fact that zebrafish were being used as a model organism. Zebrafish were used in this experiment because they have transparent eggs, making them easy to observe in lab, and have a similar genome to that of a human (Hill, Teraoka, Heideman, et al., 2005, p. 6). While using zebrafish is efficient and practical in lab, there is a chance that not all data collected from zebrafish testing will be applicable to humans.

In conclusion, the data presented in *Table 1, Figures 1-3*, and *Illustrations 4-6* supports both of the original hypotheses this lab aimed to investigate. Also, the qualitative data collected from the sucralose-exposed experimental group provided reason to form an additional hypothesis that if zebrafish embryos are exposed to sucralose, then the eggs will hatch earlier due to data collected in this experiment (*Figure 2*) that shows that the sucralose-exposed experimental group had the most hatched eggs by the early benchmark time of 48 hpf. Further testing of both the effects of saccharin and sucralose on embryonic development with larger sample sizes and more advanced model organisms may be able to provide avenues to achieve a clearer conclusion regarding how artificial sweeteners affect an organism during the developmental stages.

References

American Pregnancy Association (2015). Artificial Sweeteners And Pregnancy. *American Pregnancy Association.* Retrieved on December 18, 2018 from http://americanpregnancy.org/pregnancy-health/artificial-sweeteners-and-pregnancy/

GraphPad Software, Inc. (2016). t-test calculator. *QuickCalcs.* Retrieved from http://graphpad.com/quickcalcs/ttest1.cfm

Griglak, M. (2016). Artificial sweeteners' effect on embryological development of danio rerio. Retrieved on December 8th, 2018 from https://cpb-us-w2.wpmucdn.com/people.uwm.edu/dist/8/202/files/2016/06/Griglak_zf_Greendale-146deez.pdf

Hill, A. J., Teraoka, H., Heideman, W., et al. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences, v. 86.* 6-19. doi: 10.1093/toxsci/kfi110. Retrieved November 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/15703261

Hillman, E. T., Lu, H., Yao, T., et al. (2017). Microbial Ecology along the Gastrointestinal Tract. *Microbes and Environments*, 32(4), 300-313. doi:10.1264/jsme2.ME17017. Retrieved on December 19, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5745014/

Kim, J. Y., Seo, J., Cho, K. H. (2011). Aspartame-fed zebrafish exhibit acute deaths with swimming defects and saccharin-fed zebrafish have elevation of cholesteryl ester transfer protein activity in hypercholesterolemia. *Food and Chemical Toxicology*, 49(11), 2899-2905. doi:10.1016/j.fct.2011.08.001. Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/21855599

Marfella, R., Quagliaro, L., Nappo, F., et al. (2001). Acute hyperglycemia induces an oxidative stress in healthy subjects. *The Journal of Clinical Investigation,* 108(4), 635-636. doi:10.1172/JCI13727. Retrieved December 19, 2018 from https://www.jci.org/articles/view/13727

National Heart, Lung, and Blood Association (2014). High blood cholesterol. *Health Topics.* Retrieved December 19, 2018 from https://www.nhlbi.nih.gov/health-topics/high-blood-cholesterol

Oxbridge Solutions Ltd. (2013). t-test (paired and unpaired). *GP Notebook.* Retrieved from https://www.gpnotebook.co.uk/simplepage.cfm?ID=x20050323103508411760

Schiffman, S. S., Rother, K. I. (2013). Sucralose, A Synthetic Organochlorine Sweetener: Overview of Biological Issues. *Journal of Toxicology and Environmental Health,* 16(7), 399-451. doi: 10.1080/10937404.2013.842523. Retrieved December 18, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3856475/

Swithers, S. E. (2013). Artificial sweeteners produce the counterintuitive effect of inducing metabolic derangements. *Trends of Endocrinology & Metabolism,* 24(9), 431-441. doi: 10.1016/j.tem.2013.05.005. Retrieved December 18, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3772345/

U. S. Food and Drug Association. (2018). Additional Information about High-Intensity Sweeteners Permitted for Use in Food in the United States. Retrieved December 8, 2018 from https://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm397725.htm#Sucralose