Zebrafish Lab

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AP Biology

Abstract

Folic acid is a vital nutrient for cell division, development, and gene expression. Its importance is especially prominent in human embryonic development. The purpose of this lab was to investigate the effects of folic acid of varying concentrations on the embryonic development of Zebrafish (*Danio rerio*). Zebrafish will be used as model organisms to connect the results of the lab with folic acid exposure on human development.

Zebrafish embryos were placed in wells with a solution of either a daily dose of folic acid (scaled down for their body weight) or a solution 500x more concentrated than this. The embryos were observed daily and the stage of their development was catalogued. Then, the solutions in the wells were exchanged for fresh solution.

Based on the results of this lab, the proposed hypothesis is supported. While in the beginning stages of development the embryos exposed to 500x Folic Acid developed more quickly, they also died at a greater (but still very small) rate than the other experimental group.

When a woman becomes pregnant or expects to become pregnant, she is often advised to take folic acid supplements and to eat a folic acid-rich diet. Based on the results of this lab, this practice can be supported, due to the improved health of the embryos exposed to folic acid.

Introduction

In the course of embryonic development, many vitamins, minerals, and nutrients from the mother's diet play a key role in the proper formation of body components and functions. One such nutrient, folic acid, plays a necessary role in neural development. The purpose of this lab is to determine how folic acid in different concentrations affects the embryonic development of zebrafish (*Danio rerio*), and how this affects human embryonic development.

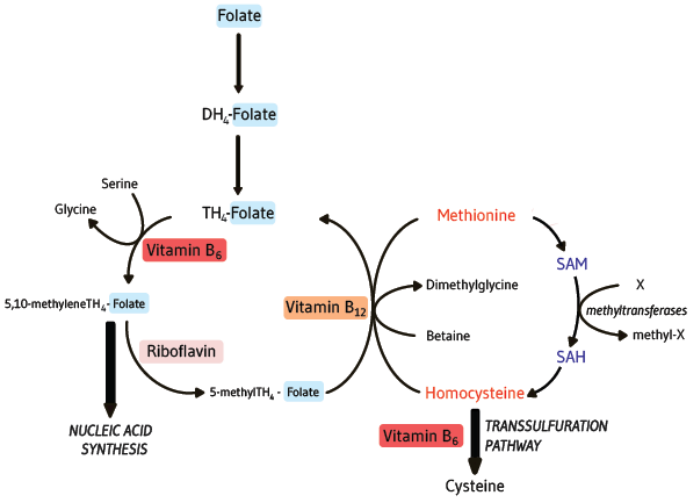

Figure 1: Folate initiates a cycle during which several intermediates, such as SAM, are created. Nucleic acid synthesis and DNA methylation are initiated.

In the folate mediated one-carbon cycle, folic acid "is a cofactor... acting as a shuttle for methyl groups that will be used in the metabolism of s-adenosyl methionine (SAM), *de novo* synthesis of purines..." (Bonner, Bernard, Sanchez, Sause, Prentice, Brody, 2012). This cycle's main functions include "purine synthesis, thymidylate synthesis and methylation reactions. There is, however, a fourth major function: the metabolism of some amino acids (serine, glycine, tryptophan and histidine), as well as choline" (Brosnan, MacMillan, Steven, Brosnan, 2015). Due to its central role in the formation and methylation of DNA, folic acid is vital for cell division, development, and gene expression. The necessity of this vitamin varies by species. Image taken from oregonstate.edu.

In humans, folic acid taken by a pregnant mother is known to prevent spina bifida and miscarriages, among other complications. In studies involving zebrafish, exposure to methotrexate, a folic acid inhibitor, has been found to increase the risk of a shorter anterior-posterior axis and cardiovascular system defects, such as incomplete cardiac looping (Lee MS1, Bonner JR, Bernard DJ, Sanchez EL, Sause ET, Prentice RR, Burgess SM, Brody LC, 2012).

A pregnant woman is advised to take 460 µg of folic acid to promote normal embryo development. The average weight of a woman in 2010 was 75.4 kilograms, compared to an average zebrafish weight of 240 micrograms. The equivalent folic acid dose in zebrafish is $1.46 * 10^{-6}$ µg. The equivalence calculations are shown below:

Daily dose of folic acid for zebrafish, based on daily values average human weight of human females (“Body Measurements”, 2016):

$$\frac{.46 \text{ mg folic acid}}{75.6 \text{ kg}} = \frac{x}{2.4 * 10^{-7}} \qquad x = 1.46 * 10^{-6} \text{ µg}$$

Amount of folic acid that must be present in a 500 mL beaker in this experiment, based on transferring 3.5 mL of solution into each well:

$$\frac{1.46 * 10^{-6} \text{ µg folic acid}}{3.5 \text{ mL solution}} = \frac{4.17 * 10^{-6} \text{ µg}}{\text{mL}} * \frac{1 \text{ mg}}{1000 \text{ µg}} \cdot \frac{1000 \text{ mL}}{1} = 4.17 * 10^{-7} \text{ mg/L}$$

$$\frac{4.17 * 10^{-7} \text{ mg folic acid}}{\text{L}} \cdot \frac{1}{2} = \frac{2.09 * 10^{-7} \text{ mg}}{500 \text{ mL}}$$

Using an 800 µg folic acid supplement, the following dilutions were completed to achieve the desired dosage:

$$.8 \text{ µg folic acid} \cdot \frac{1 \text{mg}}{1000 \text{ µg}} = .8 \text{ mg folic acid} \cdot \frac{1}{500 \text{ mL}} \cdot \frac{1}{500 \text{ mL}} \cdot \frac{1}{5 \text{ mL}} = 6.4 * 10^{-7} \text{ mg/L}$$

For the more concentrated folic acid solution, the following dilution was used, creating a solution 500 times more concentrated than the estimated daily dosage:

$$.8 \text{ mg folic acid} \cdot \frac{1}{500 \text{ mL}} \cdot \frac{1}{5 \text{ mL}} = 3.2 * 10^{-4} \text{ mg/L}$$

Zebrafish (Danio rerio) are found in the Ganges River in India. They are not a common food source nor are they instrumental in fisheries, but they are popular and well-known additions to home aquariums worldwide. Zebrafish have been used as a model organism in experiments since the 1960s. Their bodies are transparent, and they produce hundreds of offspring in one reproduction cycle. They also grow at a fast rate, making them ideal for both fast production and observation. Zebrafish also share 70% of their genes with humans, and 84% of human diseases have been found to have a zebrafish counterpart. Zebrafish are also cheaper to maintain than

other comparable model organisms, making them a cost-effective model organism when simulating human conditions.

The question of this experiment was to determine how the addition of folic acid to the environment of a developing embryo affects its development.

Hypothesis: If the appropriate estimated dose ($1.46 * 10^{-6}$ µg) is added to the zebrafish embryo environment, embryonic development will be completed more quickly and successfully than the other trials, because of folic acid's vital role in the mediated one-carbon cycle, and thus heart and neural development.

Materials and Methods

A mortar and pestle were used to crush one folic acid supplement pill into a very fine powder. Two 500 mL beakers were filled with 500 mL of water each. Another 5 mL of dechlorinated water were measured into a 100 mL beaker. The folic acid powder was then added to one of the 500 mL beakers and stirred continuously with a stir rod for several minutes. One mL of this solution was transferred to the 100 mL beaker: immediately before the solution was transferred, it was stirred quickly, to ensure that the small particles of the pill that remained were evenly distributed. The solution within 100 mL beaker was then stirred. 2.5 mL of the dechlorinated water in the remaining 500 mL beaker was removed and dumped into a sink. 2.5 mL of Instant Ocean was then added to this beaker, and the solution was stirred briefly. 1 mL of the folic acid solution in the 100 mL beaker was then added to the aforementioned 500 mL beaker. Before transfer, the folic acid solution was stirred quickly. The 500 mL beaker containing the instant ocean and folic acid was the completed solution, which the zebrafish embryos will be exposed to. This process was repeated to make the 500x concentrated solution: however, the folic acid powder was added to the 100 mL beaker, and the solution in this beaker was transferred to a 500 mL beaker only once.

A tray containing twelve 7 mL wells was used. A permanent marker was used to divide the tray in half (6 wells on each side of the tray), and to label one side as "1x Folic Acid

Solution", and the other as "500x Folic Acid Solution". Five zebrafish embryos were placed in each well using a wide bore pipette. Normal size bore pipettes were used to add 3.5 mL of one of the previously prepared solutions into each well: the solution added corresponded to the label given to each side of the tray. When the zebrafish were not being observed, they were placed in an incubator set at 28.5 °C. A stereoscope was used to observe the embryos each day, and their condition was compared to a chart which detailed the features that should be seen at each stage of development. After comparing the embryo to these images, information was added to a chart, which contained a row for every stage of development, and a column for each day the embryos were to be observed. The tally was added to the row that corresponded with the embryo's stage of development, and the column which corresponded with the day the embryo was being observed. These observations were made for four consecutive days, starting the day after the embryos were added to the wells.

A control group was not observed by the research group due to the availability of resources. Thus, control data from another group was used.

Results

The development and death rates of the embryos in the two solutions of different concentrations were observed for four days. The control group for this lab was not exposed to folic acid. The experimental groups were exposed to varying amounts of folic acid. The independent variable was the amount of folic acid the zebrafish embryos were exposed to, and the dependent variable was the rate of the fish's development. The constants included temperature of the environment, the embryos' exposure to light, their daily schedule, and the amount of Instant Ocean they were exposed to. By observing the rate of the zebrafish development, the effect of folic acid on their development can be determined.

Data Presentation

Folic Acid 1X Solution

Stage (hpf)	Day 1	Day 2	Day 3	Day 4
0.25	-	-	-	-
1	-	-	-	-
1.25	-	-	-	-
1.5	-	-	-	-
1.75	-	-	-	-
2	-	-	-	-
2.5	-	-	-	-
3.33	-	-	-	-
4	-	-	-	-
4.33	-	-	-	-
4.66	-	-	-	-
5.25	-	-	-	-
5.66	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
10.66	-	-	-	-
11.66	-	-	-	-
14	-	-	-	-
16.5	1	-	-	-
19	13	-	-	-
24	20	-	-	-
30	-	-	-	-
36	-	15	2	-
48	-	17	1	-
60	-	1	7	-
72	-	-	23	-
96	-	-	-	5
120	-	-	-	28
DEATH	-	-	-	-

Table 1: The stages of development for fish exposed to one daily dose of folic acid each day were recorded.

Folic Acid 500X Solution

Stage (hpf)	Day 1	Day 2	Day 3	Day 4
0.25	-	-	-	-
1	-	-	-	-
1.25	-	-	-	-
1.5	-	-	-	-
1.75	-	-	-	-
2	-	-	-	-
2.5	-	-	-	-
3.33	-	-	-	-
4	-	-	-	-
4.33	-	-	-	-
4.66	-	-	-	-
5.25	-	-	-	-
5.66	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
10.66	-	-	-	-
11.66	-	-	-	-
14	-	-	-	-
16.5	-	-	-	-
19	17	-	-	-
24	5	-	-	-
30	5	-	-	-
36	2	5	-	-
48	-	20	4	-
60	-	4	15	-
72	-	-	9	-
96	-	-	-	6
120	-	-	-	21
DEATH	1	-	2	-

Table 2: The stages of development for fish exposed to 500x one daily dose of folic acid each day were recorded.

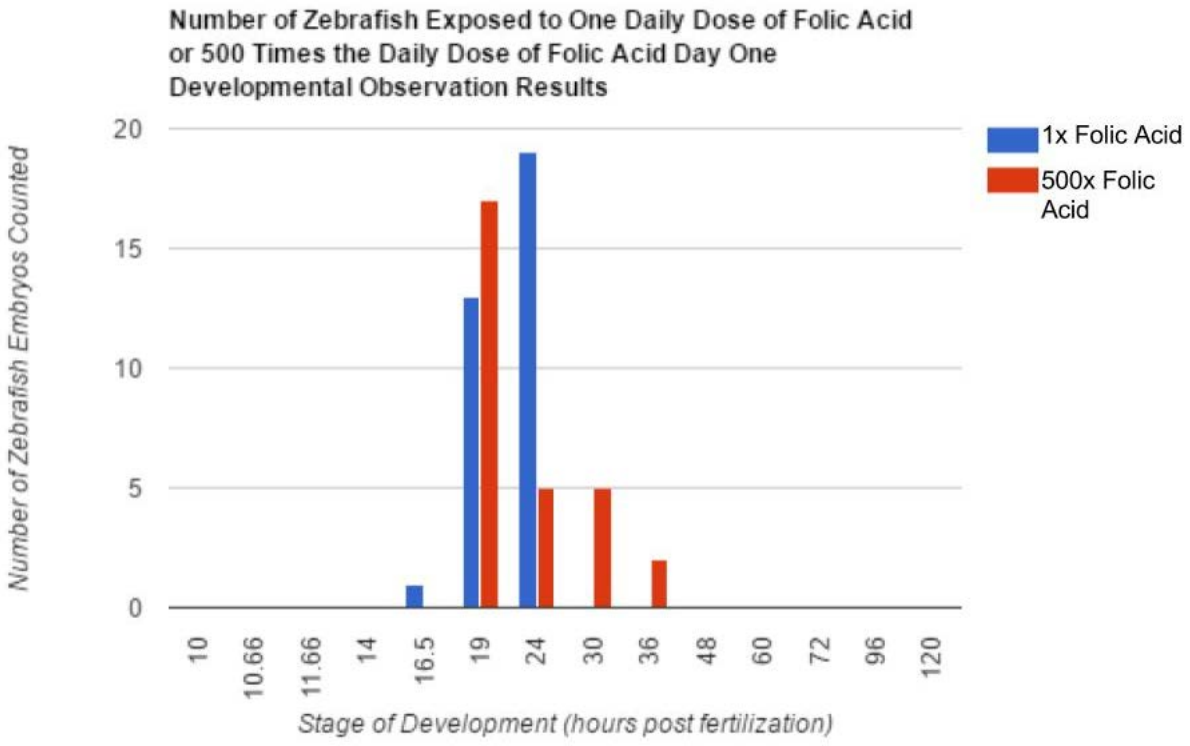

Figure 2: The number of fish that have reached each stage of development for each concentration studied on the first day of observation is shown above.

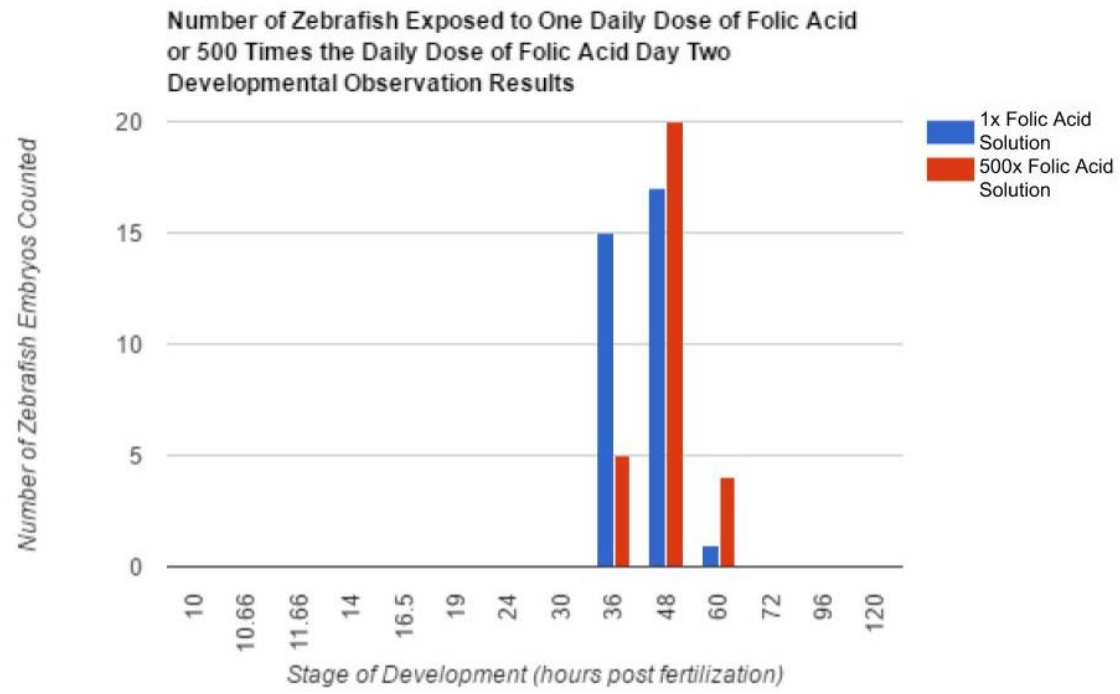

Figure 3: The number of fish that have reached each stage of development for each concentration studied on the second day of observation is shown above

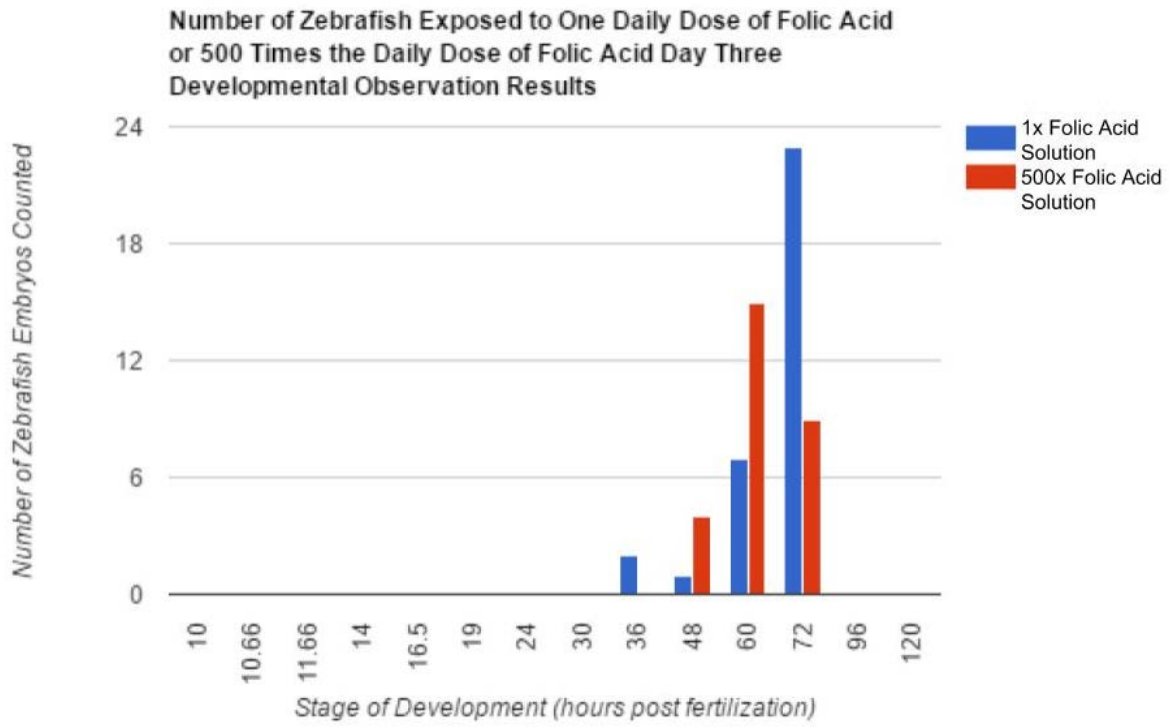

Figure 4: The number of fish that have reached each stage of development for each concentration studied on the third day of observation is shown above

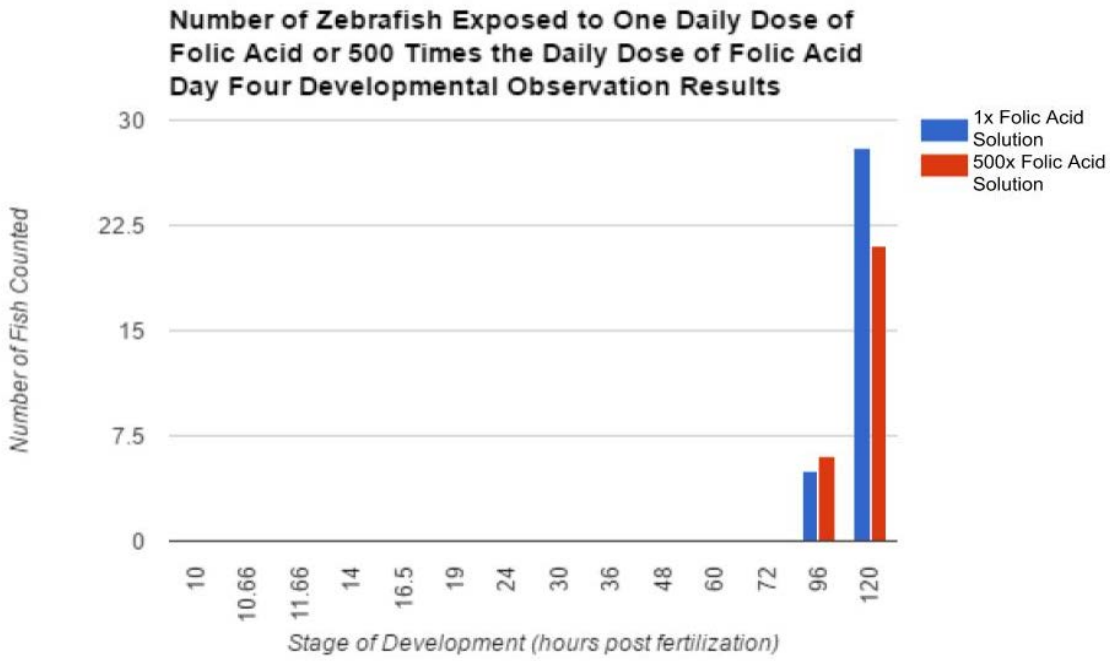

Figure 5: The number of fish that have reached each stage of development for each concentration studied on the fourth day of observation is shown above

No observations were made before 9 hours post fertilization

Day	1x Folic Acid Solution	500x Folic Acid Solution	Control
1	100	96	86
2	100	96	86
3	100	90	73
4	100	90	73

Table 3: The percentage of embryos that remained living at the end of the lab are displayed for 2x Folic Acid Solution, 500x Folic acid solution, and the control group. Due the difference in observational methods between the two research groups, different variables had to be compared.

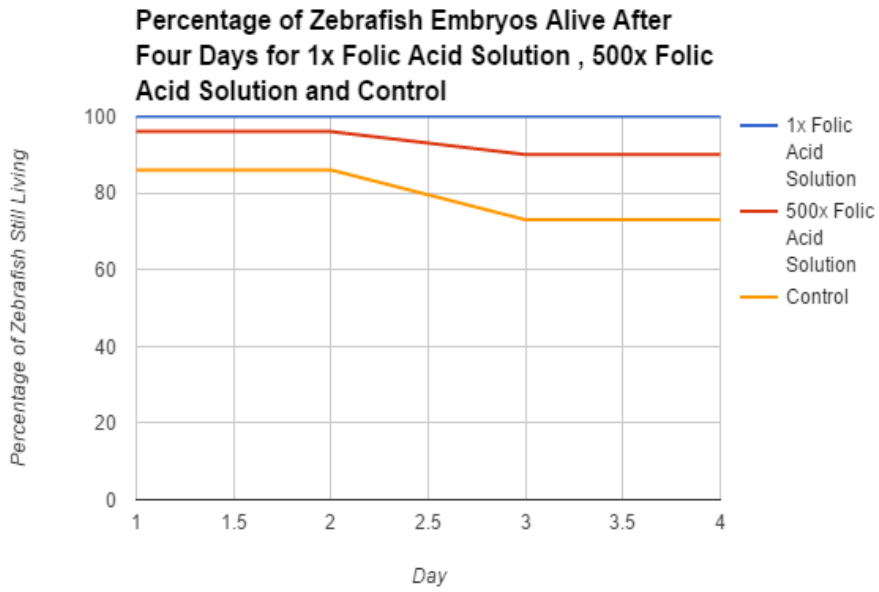

Table 3: The percentage of embryos that remained living at the end of the lab are displayed for 2x Folic Acid Solution, 500x Folic acid solution, and the control group.

The chart pictured below was used to gauge the embryos' progress. This chart was used as a control, as it details the stages of development that the embryos should reach at specific times:

Figure 6: Displays the form fish at different stages should take. This chart was used for qualitative comparison to the fish in the lab.

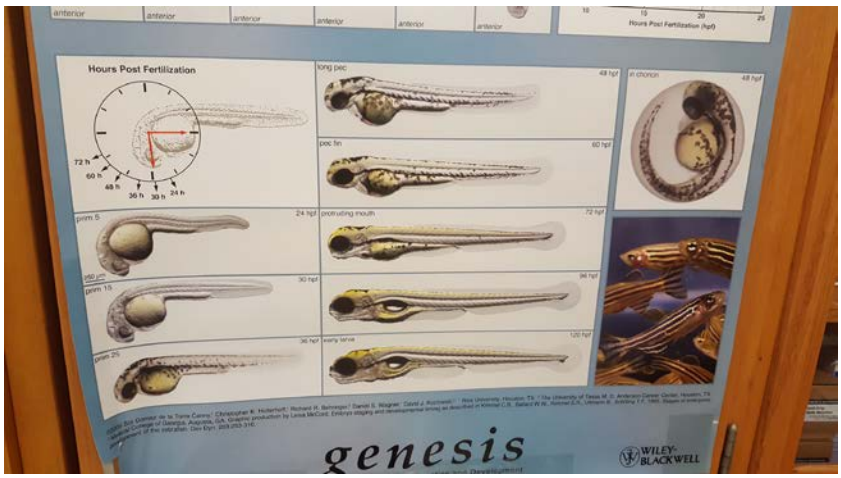

Figure 7: A continuation of the figure above.

Day	Percent of Embryos Ahead of Schedule - 1x Folic Acid Solution	Percent of Embryos Behind Schedule - 1x Folic Acid Solution	Percent of Embryos Ahead of Schedule - 500x Folic Acid Solution	Percent of Embryos Behind Schedule - 500x Folic Acid Solution
1	0	42	24	59
2	3	45	31	17
3	0	30	0	66
4	85	0	72	0

Table 4: Shows how many fish were ahead of or behind the normal development schedule (based on the chart above).

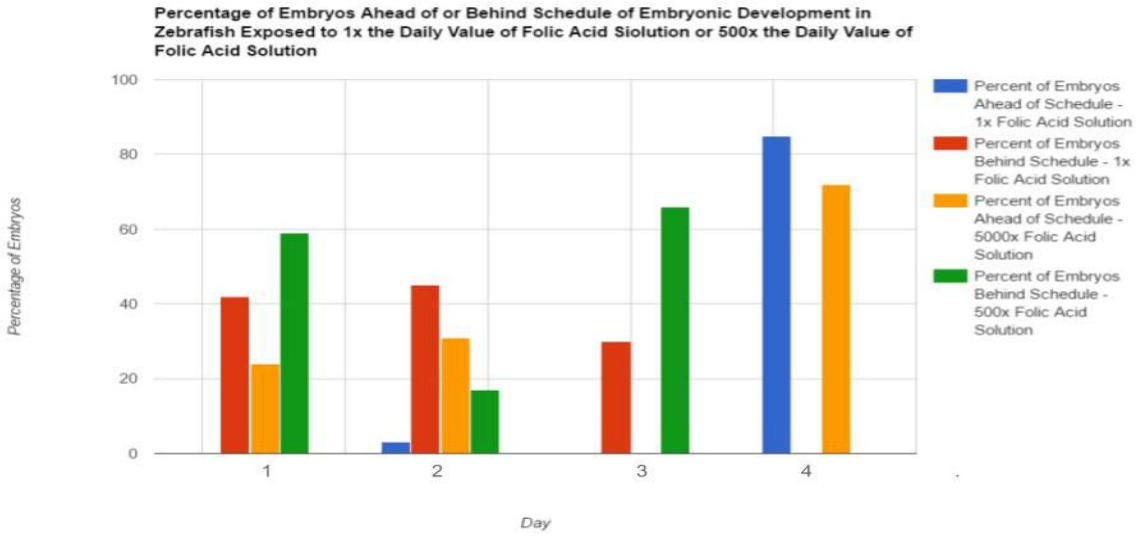

Figure 8: A graphical representation of how many fish were ahead of or behind the normal development schedule (based on the chart above).

Data Analysis

Overall, the death rates for embryos exposed to folic acid were less than the death rates for embryos not exposed to folic acid. The percentage of fish left living by the end of the lab for the control group was was 73%, while 90% of fish exposed to 500x Folic Acid and 100% of fish exposed to 1x Folic Acid survived. The death rate was greater in embryos exposed to 500x Folic Acid solution than that of embryos exposed to 1x Folic Acid Solution. For the first two days of the experiment, the embryos exposed to 500x Folic Acid Solution appeared to be developing at a greater rate than that of the embryos exposed to 1x Folic Acid Solution. This is based specifically on the percentage of embryos in each solution that were ahead of schedule on days one and two. For those in the 500x solution, the percentages of embryos ahead of schedule for these days were 24% and 31%. For the embryos exposed to 1x solution, these percentages were only 0% and 3%. On day three, this shifts, as 66% of embryos exposed to 500x Folic Acid solution were behind schedule, and only 30% of fish exposed to 1x Folic Acid were behind schedule. It should be noted, however that embryos were observed hatching as data was being recorded. By the end of the lab, embryos exposed to both solutions had reached approximately the same stages of development - 85% of embryos exposed to 1x Folic Acid and 72% of embryos exposed to concentrated Folic Acid were ahead of schedule on day four.

A t test could not be completed for the collected data, as the information being gathered (stage of development) could not be graphically represented or used to calculate values.

Discussion

Based on the results of this lab, the proposed hypothesis is supported. While in the beginning stages of development of the embryos exposed to 500x Folic Acid occurred more quickly, they also died at a greater (but still very small) rate. Additionally, the embryos exposed to 1x Folic Acid progressed at a similar pace to the embryos exposed to the more concentrated solution, suggesting a slight delay in the absorption of folic acid into the embryo. When

compared to the control group, embryos exposed to either concentration experienced superior development.

However, the results of the lab are not entirely conclusive: in future trials, the control should be set up and data should be recorded only by the researcher(s) that also set up and observe the other embryos of varying exposure. This will ensure consistency when comparing data. The exact stage of the embryos being observed could not be guaranteed, due to the qualitative methods of data acquisition. Additionally, the embryos that died may not have done so to exposure to folic acid they may have died regardless of this exposure.

When a woman becomes pregnant or expects to become pregnant, she is often advised to take folic acid supplements and to eat a folic acid-rich diet. Based on the results of this lab, this practice can be supported, due to the improved health of the embryos exposed to folic acid. Through this investigation, the benefits of folic acid in embryonic development were presented.

References

Body Measurements. (2016, July 15). Retrieved February 20, 2017, from https://www.cdc.gov/nchs/fastats/body-measurements.htm

Brosnan, M. E., MacMillan, L., Stevens, J. R., & Brosnan, J. T. (2015). Division of labour: how does folate metabolism partition between one-carbon metabolism and amino acid oxidation? Retrieved February 20, 2017, from http://www.biochemj.org/content/472/2/135

Lee MS1, Bonner JR, Bernard DJ, Sanchez EL, Sause ET, Prentice RR, Burgess SM, Brody LC. (2012)."Disruption of the Folate Pathway in Zebrafish Causes Developmental Defects." *BMC Developmental Biology.* U.S. National Library of Medicine. Web. 20 Feb. 2017.

"Folate." *Linus Pauling Institute*. 30 Jan. 2017. Web. 27 Feb. 2017.

Stover, P. J., PhD, & Caudill, M. A., PhD, RD. (2008, September). Genetic and Epigenetic Contributions to Human Nutrition and Health: Managing Genome–Diet Interactions. Retrieved from Genetic and Epigenetic Contributions to Human Nutrition and Health: Managing Genome–Diet Interactions

Why use the zebrafish in research? (2014, November 17). Retrieved February 20, 2017, from http://www.yourgenome.org/facts/why-use-the-zebrafish-in-research