Folic acid decreases the mortality rate of *Danio rerio* embryos exposed to alcohol

Tyler J. Roeder

March 22, 2018

Honors Biology

Dr. Mary Eldridge

Kohler High School, Kohler, WI
Abstract

The effects of alcohol are tragic to developing organisms. The purpose of my experiment was to determine the effectiveness of folic acid as a way to decrease the mortality rate due to the presence of alcohol. *Danio rerio* embryos were used as model organisms in the tests. It was hypothesized that, if folic acid counteracts the lethal effects of ethanol on embryos, and I expose *Danio rerio* embryos to varying concentrations of ethanol and expose half of the ethanol-exposed embryos with folic acid, then the mortality rate will be significantly lower in those exposed to folic acid. To actually conduct the test, I exposed embryos to different concentrations of alcohol and treated half of them with folic acid solution. All other variables were kept constant. Results were recorded daily, and significance tests were run at the experiment’s conclusion. Notable results included a significant difference (p=0.0001) in one experimental group that received folic acid, in comparison to its non-folate counterparts. I was able to conclude that folic acid did significantly reduce the mortality rate among alcohol-exposed zebrafish embryos. From my data, and through use of model organisms, I was able to imply that folic acid likely counteracts the deadly effects of alcohol in humans as well.

Introduction

Does folic acid help to decrease mortality in developing vertebrates?

Women who drank four or more drinks a week were found to be 2.65 times more likely to have a miscarriage compared to women who abstained [2]. More specifically, women who drank even a moderate amount during the first trimester saw an increased risk of spontaneous abortion [3]. In addition, a study on ethanol and folic acid deficiency found that low concentrations of ethanol were just as deadly as high concentrations if no folic acid is intaken. [1] This study found results that
allowed them to conclude that folic acid played a major role in counteracting alcohol’s effects on developing organisms.

So, the question was asked: Does folic acid decrease mortality rate among ethanol-exposed *Danio rerio* embryos?

Past studies have shown the negative effects of alcohol on developing organisms. Alcohol causes deaths and deformities, among other negative effects, no matter the amount. Women who drink any amount of alcohol during pregnancy put their babies at risk of death or growth issues [2].

Now, how might a person counteract the detrimental effects of alcohol? Studies have shown that folic acid decreases alcohol’s effects in other vertebrate animals, such as mice [1]. I want to know more about folic acid’s counter effects against alcohol in other animals. It worked on the mice in the previous study, but how would it work if applied to *Danio rerio* embryos or even humans? This is the knowledge gap that I attempted to fill.

It was hypothesized that: If folic acid counteracts the lethal effects of ethanol on embryos, and I expose *Danio rerio* embryos to varying concentrations of ethanol and expose half of the ethanol-exposed embryos with folic acid, then the mortality rate will be significantly lower in those exposed to folic acid.

I exposed zebrafish embryos to different concentrations (high, medium, and none) of ethanol and treated half of each section with folic acid. I counted the alive and dead embryos every day, obtaining values which I could then convert to percents or other data. I expected to obtain data that would provide evidence towards folic acid being useful as a way to counteract the effects of alcohol.
Methods

MATERIALS

Danio rerio embryos were received from the UWM School of Freshwater Sciences on the morning of February 19, 2018. The following solutions were prepared: Instant Ocean Zebrafish Medium Solution (Instant Ocean® Sea Salt + Distilled Water), 17 mM Ethanol Solution (Ethanol + Zebrafish Medium), 60 mM Ethanol Solution (Ethanol + Zebrafish Medium), 0.02 mM folic acid Solution (Folic Acid + Zebrafish Medium). Required lab supplies include a dissecting microscope, disposable pipettes, 250 mL flasks to store solutions, and a waste beaker. Overnight, a 28.5°C incubator was used to store embryos.

PROCEDURE

Proper lab safety measures (safety goggles, gloves, tight-fitting clothing) were taken at all steps of the process.

1. Before starting testing, the solutions were prepared. Instant Ocean zebrafish medium solution, 17 mM ethanol solution, 60 mM ethanol solution, 0.02 mM folic acid solution were all prepared, placed in 250 mL flasks, and capped.

2. Zebrafish Embryos were obtained from the UWM School of Freshwater Sciences. They were divided up among the six wells, with 14 control embryos, and 18 embryos for each treatment.

3. Danio rerio embryos were placed into their corresponding wells (see Figure 1), and wells were filled using a disposable pipette to a total of ~3 mL of the corresponding solution(s) (see...
4. Daily, I observed each well under the microscope, counting the number of alive and dead embryos. This data was recorded in lab notebooks. The dead embryos were then removed.
from the wells and placed into the beaker labeled “waste”. At the end of the observation period, wells were drained and refilled with the ~3 mL of new solution (Figure 2).

5. Step four was repeated for a total of three observation days until 72 hours post fertilization (hpf).

6. After 72 hours, the live zebrafish were euthanized using the WInSTEP SEPA protocol and were stained using a no-acid stain protocol and photographed (see Figure 3).

7. Fisher’s exact test (GraphPad Software Inc., San Diego, CA) was used to find statistical significance. Fisher’s test was used because of small sample sizes and due to an expected value being less than five.

**DATA ANALYSIS**

Fisher’s exact test was conducted due to small sample sizes and expected values less than 5.

Expected values were obtained from control data (13 alive and 1 dead) after three days of testing.
In cases B1, B3, and C1, significant evidence was found to reject the null hypothesis. P-Values of 0.00001, 0.00001, and 0.0189 are all less than 0.05, so I can conclude statistical significance. In A3 and C3, P-Values of 1.0000 and 0.3547 were obtained, both of which are less than 0.05. For these two wells, I fail to reject the null hypothesis, as there is insufficient evidence to reject it.

Results

A1 (control) were the values to which the other wells were compared for significance. Below, Figure 3 shows the treatment groups and the percents of zebrafish in each that fell under each category. Red bars represent alive embryos, and blue bars represent dead embryos. Table 1
displays the cumulative raw data obtained from observing embryos, at 72 hpf. (see Appendix A for all raw data).

Noteworthy Results:

1. A3: The percent of Zebrafish alive after 72 hpf were very close in both the control and the [No Ethanol + folic acid] group. No significant conclusions were drawn to support folic acid’s effects on embryos with no ethanol.

2. B1 and B3: folic acid was unable to counter the effects of ethanol on the mortality rate (all 18 embryos dead). Comparing the two values show no difference in mortality rate.

3. C3: Embryos in C3 (17 mM Ethanol + folic acid) had a survival rate of 77.7 percent. Without folic acid, embryos saw a survival rate of only 50 percent. This increase of over 25 percent came with the addition of folic acid.
Classification of Zebrafish Conditions by Treatment Type

Treatment and Time Post-Fertilization

[FIGURE 5]

Shows the percent of zebrafish that are alive and dead, hatched and alive. Shown are the results 24 hpf and 72 hpf. (see Figures 1 and 2 for key)

Raw Data (72 hpf)

<table>
<thead>
<tr>
<th></th>
<th>U-Dead</th>
<th>H-Dead</th>
<th>U-Alive</th>
<th>H-Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>B1</td>
<td>17</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>A3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>B3</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

[TABLE 1]

Shows the raw data collected (cumulative) after 72 hours of observations.
Discussion

The findings support my hypothesis: folic acid does decrease mortality rate in low concentration ethanol-exposed *Danio rerio* embryos. In the group with 17 mM ethanol and folic acid, significant decrease in mortality was found when embryos were treated with folic acid. The mortality did not decrease with the addition of folic acid in embryos exposed to high-concentration (30 mM) ethanol solution. These results suggest that folic acid does, in fact, decrease the mortality rate of zebrafish embryos exposed to ethanol. This average dose worked to help counteract the effects of low-concentration ethanol (17 mM: equivalent to one standard drink) but had insignificant effects on high-concentration ethanol (60 mM: equivalent to binge drinking). These results suggest that a regular dose of folic acid would help to counteract small amounts of alcohol in a pregnant woman. However, due to my results, I am unable to make a conclusion about whether a higher dose of folic acid would be more effective against high-concentration ethanol. A higher dose may or may not be effective in helping women who drink more, but I am unable to decide that due to my results.

The results of this study match up with the findings of Gutierrez’s team in a 2007 experiment with ethanol and folic acid[1]. The presence of folic acid had beneficial results on their model organism (Swiss mice).

Possible errors during this study that may have influenced results include small sample sizes and varying overnight incubator temperatures.

Other investigations that may be interesting based on the results of my experiment include effects of different folic acid doses and folic acid’s effect on the longevity of life.

In conclusion, the data obtained made sense in the big picture, and significant results were
found to support my hypothesis. It makes sense that folic acid decreased the mortality rate of low-concentration ethanol-exposed, as previous experiments convey similar ideas. Since all 18 zebrafish in each high ethanol well died, I concluded that the given amount of folic acid did not help to reduce mortality in these groups. That is not to say folic acid would not help women who drink a lot, but a larger dose may be needed. I can generalize my results to pregnant women, as the experiment used zebrafish as models for understanding the effects of ethanol on human health while in-vitro.

All pregnant women should take folic acid supplements during their pregnancy. The average dose of folic acid that I used in my experiment worked to counter the negative mortal effects of [the equivalent of] even one standard drink a day [3]. If no alcohol is consumed, consuming folic acid will not have a negative effect on the survival of babies, and it is a good idea to take supplements as a way to prevent other problems, including those involved with alcohol, during pregnancy. A person who drinks more than one drink may need a slightly higher dose of folic acid, an exact amount that could be determined by further testing.
Literature Cited


Appendix A

Daily Data (Non-Cumulative)

<table>
<thead>
<tr>
<th></th>
<th>0 hpf</th>
<th></th>
<th>24 hpf</th>
<th></th>
<th>48 hpf</th>
<th></th>
<th>72 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U-Dead</td>
<td>H-Dead</td>
<td>U-Alive</td>
<td>H-Alive</td>
<td>U-Dead</td>
<td>H-Dead</td>
<td>U-Alive</td>
</tr>
<tr>
<td>A1</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>17</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>A3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>B3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>C3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Cumulative Data

<table>
<thead>
<tr>
<th></th>
<th>0 hpf</th>
<th></th>
<th>24 hpf</th>
<th></th>
<th>48 hpf</th>
<th></th>
<th>72 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U-Dead</td>
<td>H-Dead</td>
<td>U-Alive</td>
<td>H-Alive</td>
<td>U-Dead</td>
<td>H-Dead</td>
<td>U-Alive</td>
</tr>
<tr>
<td>A1</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>17</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>A3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>B3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>C3</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>