The Effect of Sodium Benzoate on the Survival & Hatch Rates of Zebrafish Embryos

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Abstract

In recent years, zebrafish, known by the scientific name *danio rerio,* have been used more and more in a vast array of scientific studies. Most studies relating to zebrafish are focused on the effect that different toxicants have on them. The positives to using zebrafish as a model for humans include the fact that their genome has been sequenced, the fact that they possess 70% of human genes, reproduce very quickly and easily, are extremely easy to take care of, and the development of their embryos are very similar to the development of human embryos. While this study did not have the technology to study the effect of sodium benzoate on the genes of zebrafish embryos, it did look at how sodium benzoate, a possibly dangerous preservative used commonly in soda, affected the survival and hatch/developmental rates of zebrafish. The study concluded that exposure to higher concentrations of sodium benzoate led to earlier and more rapid decreases in the survival rate of zebrafish. It also concluded that sodium benzoate affected the hatch rate of zebrafish embryos. All of the concentrations of sodium benzoate used in the experiment (500 ppm, 750 ppm, 1000 ppm) were at or below the FDA limit of 0.1%, or 1000 ppm.

Introduction

There are several reasons that zebrafish, which are also known as *danio rerio*, are commonly used as a model for human development[2]. Zebrafish possess many of the same organs as humans and their organs function and respond to toxicity in similar ways to humans[2]. Moreover, zebrafish possess 70% of human genes and their genome has been sequenced[1 & 2]. Zebrafish also have a much more rapid development from embryo to adult and zebrafish studies have a lower cost of supplies and maintenance when compared to other commonly studied animals, such as mice[1, 2, & 10].

Sodium benzoate, which is known by the chemical formula $C_7H_5NaO_2$, is used as an antimicrobial preservative and flavoring agent in the food industry and as a tablet and capsule lubricant in the pharmaceutical industry[8]. Although sodium benzoate is

used in many foods and beverages in the food industry, it is most commonly used in soft drinks like Mountain Dew and Dr. Pepper[4, 7, & 8]. When sodium benzoate is mixed with ascorbic acid, which is also known as vitamin C, they can form the carcinogen benzene[4]. However, benzene's FDA approved limit in water is 5 parts per billion, and studies have shown that the presence of sodium benzoate and vitamin C in soft drinks rarely comes close to exceeding that number[4].

However, sodium benzoate has been linked to an increase in reported ADHD and low-grade inflammation throughout the body[4 & 5]. In a genotoxicity study, sodium benzoate was also reported to increase DNA damage in cultured human cells[5]. In one study on the effect of sodium benzoate on zebrafish embryos, it was shown that sodium benzoate is able to induce neurotoxicity and nephrotoxicity in zebrafish larvae[9]. The FDA approved limit in food products is 0.1% (1000 ppm)[3]. That same study reported no deaths induced by sodium benzoate in concentrations under 1000 ppm[9]. However, a separate study reported several deaths induced by sodium benzoate in concentrations lower than 1000 ppm[3]. Despite contrasting survival rate results, both studies reported neurological and physical malformations in concentrations under 1000 ppm[3 & 9]. If zebrafish embryos are placed in solutions with concentrations of sodium benzoate that are below or at the FDA approved limit (500ppm-750ppm-1000 ppm), zebrafish hatch rates and zebrafish survival rates will be noticeably higher than the corresponding rates of the control group.

Materials List

- 1 bottle for each stock solutions of sodium benzoate (500, 750, 1000 ppm)
- 1 bottle of Instant Ocean stock solution
- 1 sharpie
- 1 beaker for dead embryos and liquid disposal
- 2 plates with wells
- 1 28.5°C incubator
- 1 compound microscope
- 1 disposable 1 mL pipette for transferring embryos and changing the solution of each of the four stock solutions

Procedure

Day 1:

1. Obtain rinsed embryos.
2. Label the plates with your name and label the sodium benzoate concentration of each well with a sharpie.

3. Fill four wells with Instant Ocean and four wells with 500 ppm sodium benzoate solution in plate 1. Fill four wells of plate 2 with 750 ppm sodium benzoate solution and four other wells of plate 2 with a 1000 ppm sodium benzoate solution. Use a different disposable pipette for each solution. Place 10 fertilized embryos in each well.*
4. Place each plate in the 28.5°C incubator.

Day 2:

1. Remove the plates from the incubator.
2. Remove any dead embryos from wells and squirt them into a waste beaker using disposable pipettes.
3. Count remaining embryos, hatched fish, and record in the data table.
4. Remove the solutions from each well.
5. Refill the wells with the same stock solution that they previously had using clean pipettes for new solutions.
6. Place each plate under the compound microscope and record observations on the student data sheet.
7. Return the plate to the appropriate 28.5°C incubator

Day 3:

1. Repeat Day 2 work and observations. Record all data.

Day 4:

1. Repeat Day 2 work and observations. Record all data.

Day 5:

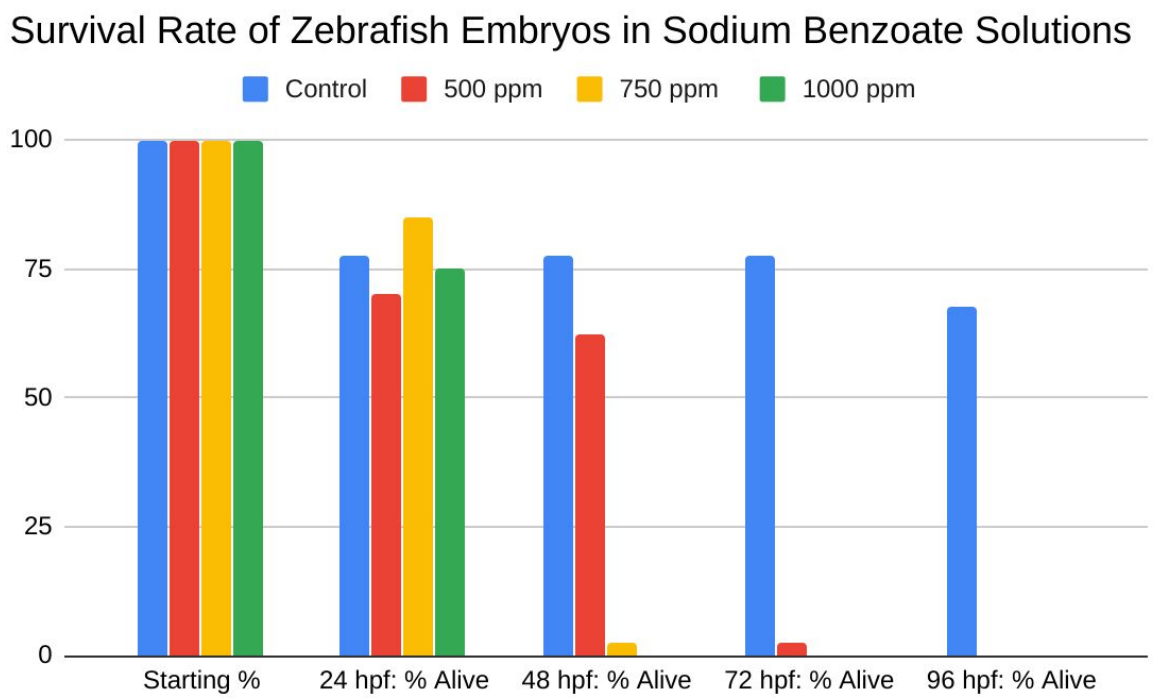
1. Repeat Day 2 work and observations. Record all data.
2. Dispose of all remaining embryos and fish in a waste container.

*Due to an error by the research team, only five zebrafish embryos were placed in the wells of the 750 ppm and 1000 ppm groups. Because of this, all data is shown as percentages.

Above procedure was taken from: SEPA Program- UW- Milwaukee[6]

Data

Figure 1



Data Table 1- Survival rate of Zebrafish Embryos in Sodium Benzoate Solutions

Time	Control	500 ppm	750 ppm	1000 ppm
Starting %	100	100	100	100
24 hpf: % Alive	77.5	70	85	75
48 hpf: % Alive	77.5	62.5	2.5	0
72 hpf: % Alive	77.5	2.5	0	0
96 hpf: % Alive	67.5	0	0	0

Data Table 2- Survival Rate P-Values

Comparison	P-value	Statistical Significance
Control vs 500ppm-24hpf	0.2782	Not significant
Control vs 750ppm-24hpf	0.5098	Not Significant

Control vs 1,000ppm-24hpf	0.7304	Not significant
Control vs 500ppm-48hpf	0.1428	Not Significant
Control vs 750ppm-48hpf	<0.0001	Extremely Significant
Control vs 1,000ppm-48hpf	<0.0001	Extremely Significant
Control vs 500ppm-72hpf	<0.0001	Extremely Significant
Control vs 750ppm-72hpf	<0.0001	Extremely Significant
Control vs 1,000ppm-72hpf	<0.0001	Extremely Significant
Control vs 500ppm-96hpf	<0.0001	Extremely Significant
Control vs 750ppm-96hpf	<0.0001	Extremely Significant
Control vs 1,000ppm-96hpf	<0.0001	Extremely Significant

*There were 40 fish in the control and 500 ppm groups to start. Due to an error in setup, there were only 20 fish in the 750 and 1000 ppm groups to start.

All of the groups had similar survival rates at the 24 hours post-fertilization mark, but all of the fish in the 1000 ppm group died before the 48-hour mark. All but one of the fish in the 750 ppm group also died before 48 hours. Some of the 500 ppm group also died in that period, but not nearly as many as died in the higher toxicant groups. By 72 hours, the 750 ppm group was fully dead and there was only one survivor in the 500 ppm group. The last survivor was dead by the 96-hour mark. The control group did not lose any fish from the 24-hour mark to the 72-hour mark, but it experienced a small dieoff at the 96-hour mark.

After 24 hours, there was no observable difference between the control group and the other groups. After 48 hours, there was no statistical significance between the control group and 500 ppm groups' survival rates. However, the difference of the survival rates between the control group and the two higher toxicant groups was extremely significant. After 72 hours and beyond, the difference in the survival rates of the control group and all of the groups exposed to toxicants were extremely significant.

Figure 2- 500 ppm Group (72 hpf)

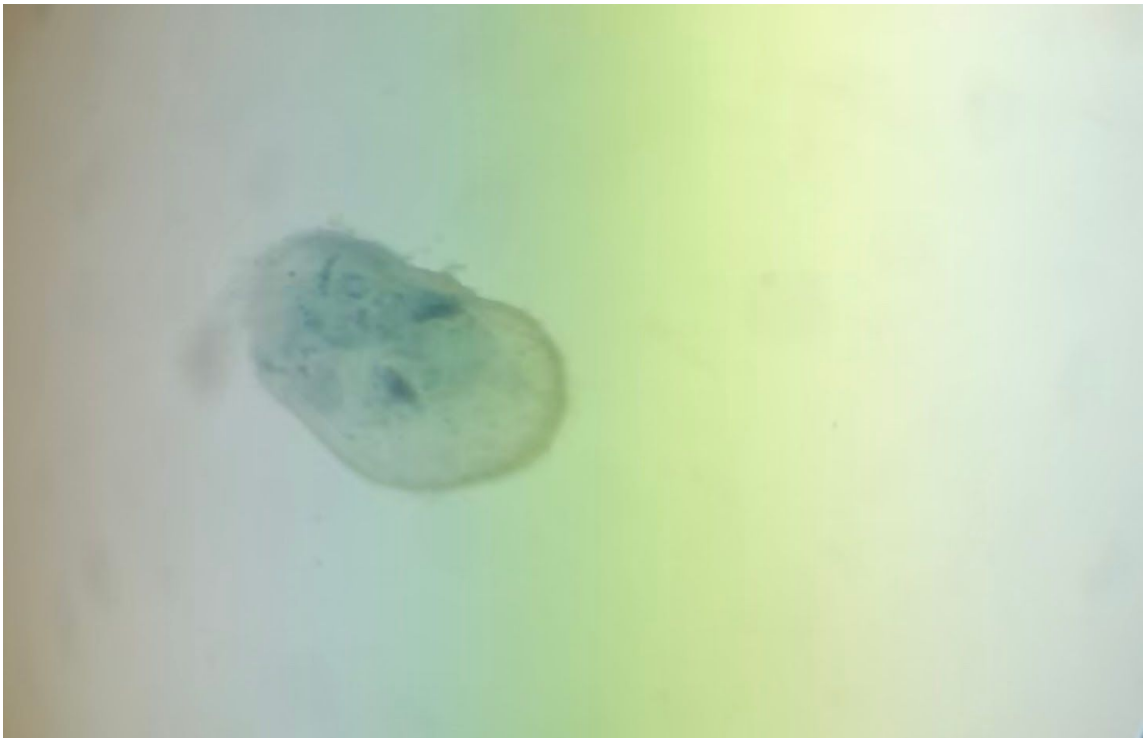
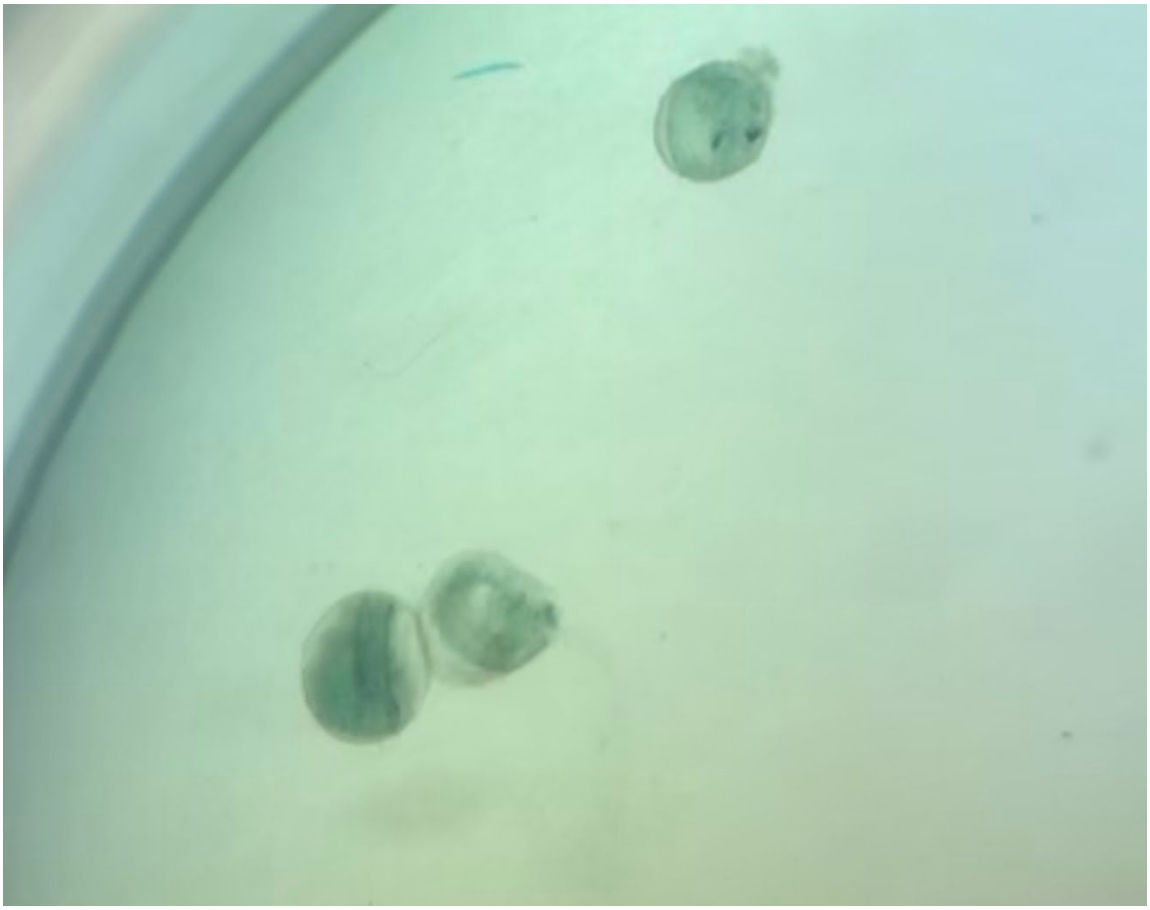
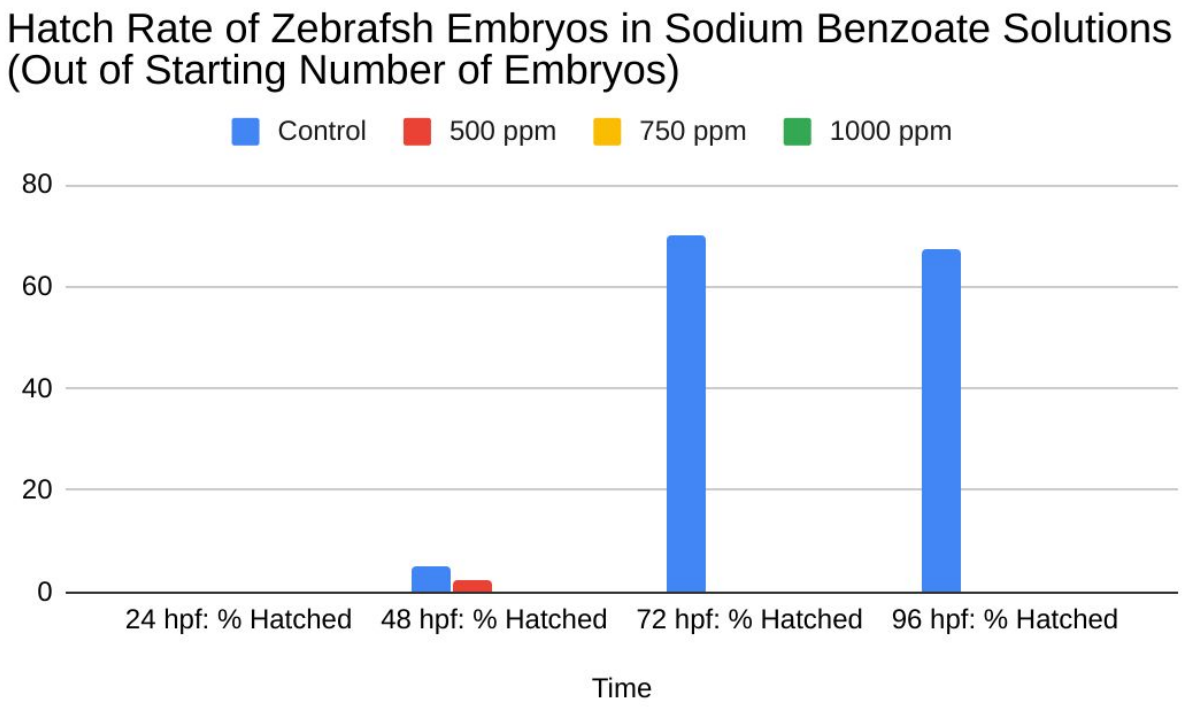


Figure 3- 500 ppm group (72 hpf)



Figures 2 & 3 both show dead zebrafish embryos from the 500 ppm group at the 72 hours post-fertilization checkpoint.

Figure 4



Data Table 3- Hatch rate of Zebrafish Embryos in Sodium Benzoate Solutions (Out of Starting Number of Embryos)

Time	Control	500 ppm	750 ppm	1000 ppm
24 hpf: % Hatched	0	0	0	0
48 hpf: % Hatched	5	2.5	0	0
72 hpf: % Hatched	70	0	0	0
96 hpf: % Hatched	67.5	0	0	0

Data Table 4

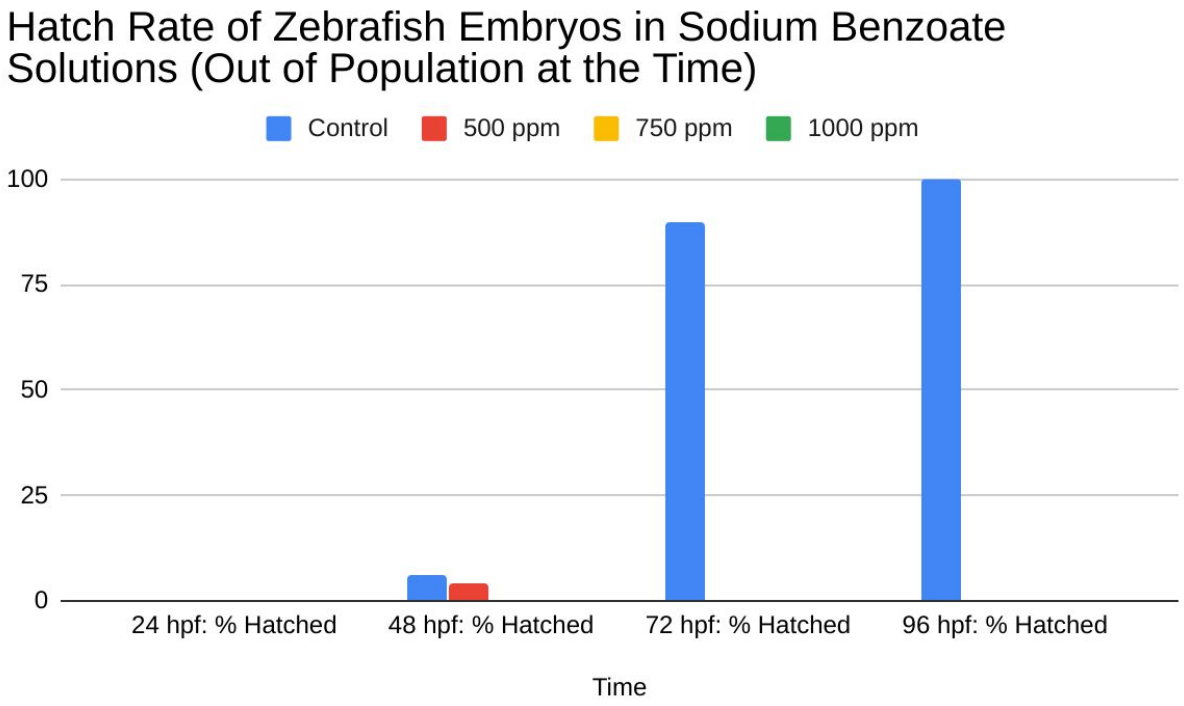
Hatch Rate P-Values (Out of Starting Numbers)

Comparison	P-value	Statistical Significance
Control vs 500ppm-24hpf	1.0000	Not significant
Control vs 750ppm-24hpf	1.0000	Not Significant
Control vs 1,000ppm-24hpf	1.0000	Not significant

Control vs 500ppm-48hpf	0.5370	Not Significant
Control vs 750ppm-48hpf	0.1340	Not Significant
Control vs 1,000ppm-48hpf	0.1340	Not Significant
Control vs 500ppm-72hpf	<0.0001	Extremely Significant
Control vs 750ppm-72hpf	<0.0001	Extremely Significant
Control vs 1,000ppm-72hpf	<0.0001	Extremely Significant
Control vs 500ppm-96hpf	<0.0001	Extremely Significant
Control vs 750ppm-96hpf	<0.0001	Extremely Significant
Control vs 1,000ppm-96hpf	<0.0001	Extremely Significant

*Figure 4 and data tables 3 and 4 show and use percentages out of the starting number of fish hatched, not as the percentage of the fish hatched that were alive at the time.

Figure 5



Data Table 5- Hatch rate of Zebrafish Embryos in Sodium Benzoate Solutions (Out of population at the Time)

Time	Control	500 ppm	750 ppm	1000 ppm
24 hpf: % Hatched	0	0	0	0
48 hpf: % Hatched	6	4	0	0
72 hpf: % Hatched	90	0	0	0
96 hpf: % Hatched	100	0	0	0

No zebrafish embryos had hatched at the 24-hour mark. All of the zebrafish embryos in the 750 and 1000 ppm groups died before they developed beyond the embryonic stage or hatched. At 48 hours post-fertilization, the control and 500 ppm groups had similar hatch rates, but the dieoff that the 500 ppm group experienced between the 48 and 72-hour marks led to its hatch rate falling back to 0%. Meanwhile, 72 hours post-fertilization, the control group's hatch rate rose to 70% of its starting number of fish (28/40) and 90% of the fish that were still living at the time (28/31). By 96 hours post-fertilization, due to a small dieoff, the control group's hatch rate had fallen to 67.5% of the starting number (27/40). However, the hatch rate was 100% of the population at the time (27/27). Due to the high death rate experienced in the groups exposed to sodium benzoate, the hatch rate was not easily observed.

The research team decided to use unpaired t-tests to find the statistical significance of the results. An unpaired t-test compares the means of two distinct groups such as the comparing of the control group to the 500 ppm group.

Results

At 24 hours post-fertilization, the survival rate of the control group was lower than the survival rate of the 750 ppm group and only 2.5% higher than the survival rate of the 1000 ppm group. It was also only 7.5% higher than the survival rate of the 500 ppm group. All of these data comparisons were determined to not be statistically significant by an unpaired t-test. From that point on, the survival rates of the 750 ppm and 1000 ppm groups plummeted. The 500 ppm group experienced a similar drop in survival rate a day later, at the 72-hour mark. By 96 hours post-fertilization, the only living zebrafish were in the control group. The 750 ppm group and 1000 ppm group were both fully dead before any of the embryos hatched. The 500 ppm group had one hatched zebrafish at 48 hours post-fertilization and the control group had two. While the hatched zebrafish in the 500 ppm group died before 72 hours, the hatch rate of the

control group rose dramatically. When the hatch rate of the control group was compared to the hatch rates of all other groups at the 72 and 96-hour marks via an unpaired t-test, the data was extremely statistically significant.

Discussion

Overall, at 24 hours post-fertilization, there was little to no difference between any of the groups in either survival rate or hatch rate. However, the 750 ppm and 1000 ppm groups' survival rates plummeted at 48 hours post-fertilization. From that point on, the difference between the control group's survival rate and the survival rates of the two largest toxicant groups was very large. The group exposed to the smallest concentration of sodium benzoate, the 500 ppm solution, had a very similar dieoff. However, its dieoff came a day later. That fact, coupled with the fact that the 750 ppm group had one survivor at 48 hours post-fertilization and the 1000 ppm group had no survivors, further confirms sodium benzoate's effects on the survival rate of zebrafish embryos by showing a correlation in the decline of survival rate and the concentration of the sodium benzoate solution that the embryos were exposed to.

The hatch rates of the zebrafish embryos, the second dependent variable in the study, is much harder to be sure about than the survival rate. While data tables 3, 4, and 5 and the unpaired t-test show extremely significant data in the comparison of the hatch rates of the control group and the toxicant-exposed groups at 72 and 96 hours post-fertilization, that is mostly due to the fact that all of the zebrafish in the toxicant-exposed groups were dead at that time. While the research team observed that the dead zebrafish embryos did seem to be underdeveloped when compared to the other embryos and hatched zebrafish, there was a lack of significant supporting evidence.

Technically, the hypothesis that hatch rates and survival rates would be significantly lower in toxicant-exposed groups was supported. In the future, conducting a very similar experiment with different toxicant levels could help to better study the dependent variable of hatch rates and to confirm the correlation of the survival rate to the concentration of sodium benzoate that zebrafish are exposed to. This experiment would use 500 ppm as the highest toxicant level and replace the other two toxicant groups with sodium benzoate stock solutions of 250 ppm and 125 ppm.

This study's results matched those of one previous zebrafish embryo study on sodium benzoate[3] by showing a dramatically decreased survival rate in zebrafish groups exposed to levels of sodium benzoate below or at the allowed FDA limit of 1000 ppm[4]. A third study, however, did not show an effect on the survival rate of zebrafish embryos exposed to sodium benzoate solutions of 1000 ppm or less[9]. The

study that was suggested above, which would use sodium benzoate solutions of 125, 250, and 500 ppm, would help to affirm whether or not levels of sodium benzoate below the FDA approved limit do have an effect on the survival rate of zebrafish embryos. While an increased mortality rate might not be an on humans who consume sodium benzoate, an increased mortality rate in zebrafish embryos is one of the most clear-cut, quantitative ways to measure the negative effects of sodium benzoate and be an indicator that sodium benzoate could have other smaller, but still harmful effects on humans. Sodium benzoate's most popular use is in soft drinks, particularly sodas[4 & 7]. Several of the most popular sodas in America, from Mountain Dew to Fanta and some Dr. Pepper varieties, list sodium benzoate as an ingredient[7].

With the incredible popularity of soft drinks both in America and around the globe, and the few studies that have looked at sodium benzoate often reporting negatively affected mortality rates along with neurological and physical malformations[3 & 9], sodium benzoate could very well be detrimentally affecting millions of people and there should be a serious reconsideration of the FDA allowed limit of sodium benzoate.

Citations:

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