The Effect of PTU on the Development of Zebrafish Embryo

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Abstract

This experiment aimed at finding the potentially harmful properties of the chemical PTU (phenylthiourea) by comparing the development of zebrafish in a standard instant ocean solution against a 0.005% PTU instant ocean solution. The data collected included the survival rate, hatched percentage, and the pigmented percentage of the zebrafish populations over the first 96 hours post-fertilization. It was hypothesized that the presence of PTU would lead to a smaller pigmentation percentage because of its property of inhibiting tyrosinase, an enzyme used in melanogenesis. Zebrafish embryos were divided into 6 wells, three exposed to PTU, and they were monitored each day up until 96 hours post-fertilization. An unpaired t-test determined that there was a statistically significant difference in hatching percentage and pigmentation percentage between the two groups (p-value < 0.0001), but the difference in survival rate was not found as significant ($p = 0.1081$). After 48 hours, the survival rate in PTU exposed populations diverged from control populations as it decreased, eventually reaching total mortality by 96 hours post-fertilization. Additionally, while zebrafish developed in the control populations with most of them hatching and gaining pigmentation, no fish in the PTU populations hatched or gained pigmentation. The results support that the presence of PTU inhibits the production of pigmentation, as well as suggesting it affects other areas during development leading to a lower hatching percentage.

Background Information

Zebrafish as a Model

Zebrafish are a great model animal for many reasons. The results of experimentation on zebrafish translate well to human beings because of their shared genetic similarities, including the fact that humans share roughly 70 percent of the same genes as zebrafish (Burke, 2016). Therefore, once a variable's effect on a zebrafish is determined, it can be reasonably assumed that it will have a similar effect to a person. Zebrafish have other benefits as well: they're less expensive to maintain and obtain compared to mice, another potential model animal, and their translucent external eggs provide ideal observation conditions during embryonic development, which were valuable attributes for our experiment (Fiaschetti & Manner).

PTU/Phenylthiourea

The experimental variable for this experiment was exposure to PTU through the usage of a solution containing a concentration of 0.005% PTU. Many experiments have been conducted on PTU supporting that it inhibits melanogenesis, the process that produces melanin. Melanin is known as the protein responsible for pigmentation in animals, so therefore, by inhibiting its production, PTU stops the creation of pigment. More specifically, PTU inhibits its production by covalently bonding with the enzyme Tyrosinase, which is essential to melanogenesis. Through the bonding of PTU with the enzyme, Tyrosinase experiences a loss in function, halting the process altogether (Hall & Orlow, 2005). Additionally, PTU, like many chemicals, is toxic and harmful in large quantities.

An investigation done by Karlsson, von Hofsten, and Olsson in 2001 aimed to determine the parameters of the drug PTU. They used a similar experiment to confirm PTU's property of inhibiting melanogenesis, and they specifically point out that the drug doesn't remove pigment already produced, so it needs to be introduced in the embryonic period. They establish the drug

as a toxin when it is exposed in too great of quantities. Their research aimed at finding an ideal concentration for creating transparent fish, and they focused on attributes similar to this experiment, such as the amount of pigmentation and survival rate of the fish.

The experimental variable chosen for this study was the presence of PTU because its effects, such as a lack of pigmentation, are easily observed with our available equipment. By building the known understanding on the chemical makeup of PTU, and furthering the knowledge on how it inhibits the enzyme tyrosinase, we would like to learn more about the specific pathway of melanogenesis. Ideally, our research would prove valuable as a potential gateway to more information in melanogenesis. Understanding how the pathway of melanogenesis functions is very important since there are genetic disorders like albinism which result from a mutation negatively affecting this pathway, and albinism can lead to problematic health conditions such as skin cancer. Knowing more about one limitation of this pathway and the enzyme tyrosinase can help us draw connections with other limitations that lead to disorders like albinism, and potentially lead to solutions that arise from albinism.

The Investigation

It is accepted that PTU inhibits the production of pigmentation; however, there are specific parameters and underlying issues with exposure to PTU that have yet to be determined, such as developmental issues could be harmful. Through the usage of zebrafish as a model, we investigated the potentially harmful effects of PTU on development by exposing it to populations of embryo and comparing any changes in pigmentation percentage, survival rate, and hatching rate. We hypothesize that the presence of PTU will inhibit the production of melanin because of its inhibiting effect on tyrosinase, which is a key enzyme in the process of creating melanin. Additionally, we hypothesize that the presence of PTU will have enough of a harmful effect to lower hatching rates and survival rates of the embryo because it could negatively impact an

undetermined but necessary component in development. After monitoring the statuses of the control group and the PTU exposed group for 96 hours post fertilization, the trend in our data suggests PTU decreases pigment and negatively impacts hatching rates.

Materials and Methods

Participants

Students of AP Biology at Greendale Highschool ran experiments to see the effect of chemicals on zebrafish embryos. Zebrafish which were bred from The School of Freshwater Sciences, at the University of Wisconsin Milwaukee. We received the fish within a couple of hours of fertilization and began our experiment on the same day. We conducted research to determine the potential effects of multiple drugs on the development of the zebrafish, with this experiment focusing on the drug phenylthiourea.

Materials

Zebrafish

The zebrafish used were bred from The School of Freshwater Sciences, at the University of Wisconsin Milwaukee. They were received within a couple of hours of fertilization and experimentation began on the same day.

Solutions

The controlled solution we used for our fish was instant ocean. This solution is often used for keeping zebrafish since it is manufactured under specific conditions to closely mimic controlled sea water. This solution is used as a standard for quality in many scientific research facilities and aquariums, bolstering its effectiveness as a control (Orr, 2008).

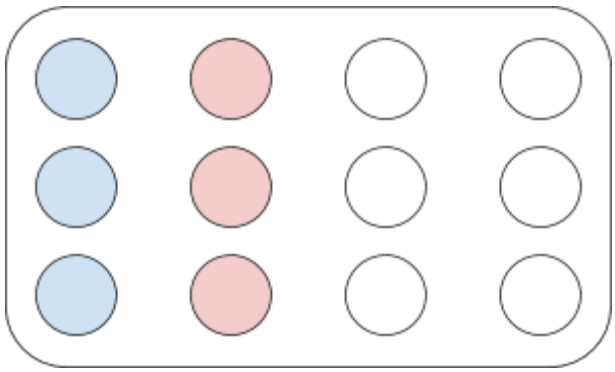
The chemical we exposed to the experimental group of fish was PTU (phenylthiourea). Our school received a 50 mL solution of 0.5% PTU, which we diluted into 40 mL of a 0.005% PTU solution with instant ocean for our experimental purposes. The PTU solution we diluted was consistent for our experimental group throughout the experiment.

Lab Equipment

To separate our populations of zebrafish, we used a 12-Well plate. We used a total of six wells, with three having the instant-ocean solution and the other three have the 0.005% PTU solution with instant ocean. To observe the zebrafish, we used a stereo microscope that allowed us to visually determine whether or not a zebrafish was alive and whether or not it had pigmentation. We used an incubator to provide a controlled and protected environment for the embryo overnight. We used two separate types of disposable pipettes. A wider nose pipette was used for egg transportation (usually for removing dead embryos). A thinner nose pipette was used for solution removal and replacement.

Design

Figure 1: 12-Well plate set-up to isolate populations.



Isolated Populations:

We isolated 6 separate populations by using one well for each. The first column of wells *indicated by blue* was dedicated to populations with the instant ocean solution (control group). The second column of wells *indicated by red* was dedicated to populations exposed to PTU (experimental group).

To determine whether or not PTU had an effect on the development of zebrafish embryos, we had to isolate two groups of zebrafish, totaling 6 different populations, to make a comparison.

Each day, by counting the amount alive in each well as well as the total, we could determine the survival rate, and with this design, we could determine which solution it fell under.

We determined that an embryo was dead by a split yolk that resulted in a dark foggy color around the entire insides of the egg. We determined a fish was “hatched” by it having it’s entire tail outside of its eggshell (we had no contestable instances in this experiment). We also measured whether or not a zebrafish was pigmented, which we defined as “containing significant dark coloring disregarding the eyes or yolk (which were slightly dark regardless of presence of melanin).”

Having the fish in the same tray of wells was also part of the design since it allowed for as much as an equally controlled environment as possible because, outside of the experimental variable, they were exposed to the nearly same amount of environmental variables such as shaking, light, temperature, etc.

Variables

The presence of the chemical PTU was changed to see if there would result in a differing measurement for any of our dependent variables. The independent variable was whether or not PTU was present in the instant ocean solution. The dependent variables were the survival rates of the fish, the percentage of fish that hatched, and the percentage of fish that developed pigmentation. There were many controls, but a few of them were the temperature of the surroundings, the amount of time inside the incubator, the amount of fluid in each well (2-3 mL), and the length of time between solution renewal (24 hours). Controls are kept constant throughout the experiment to ensure that the cause/effect relationship we are observing is truly accurate.

Procedure

1. Create two seperate 50 mL solutions, one entirely of instant ocean, and one that contains 0.005% PTU in its concentration.

2. Label the first column of wells (3 total) as “control”, and the second column of wells (3 total) as “PTU”. Then, insert roughly 8 zebrafish eggs into each well using wide pipettes.
3. Using thin pipettes, replace the older solution in each well with a fresh solution of its designated type (either control or PTU: determined by label) while keeping the eggs in the well. Then, use the microscope and document the total number of eggs in each well, the number pigmented, the number that was dead, and the number that was hatched. Pigmentation was determined by our definition: “containing significant dark coloring disregarding the eyes or yolk.” A hatched fish was determined by our definition: “the tail was completely outside of the eggshell.”
4. Using wide pipettes, remove any of the eggs that were identified as “dead”.
5. Take photos for each day, and label them with relevant information regarding the qualities of the fish (alive/dead, pigmented/albino), their well located (ex. 1A), the time taken (ex. 48 hours post-fertilization), and treatment given (ex. control).
6. Place the well-dish in an incubator overnight for the embryos' protection.
7. Repeat steps 3-6 once every day, until 96 hours post-fertilization occured (last day of recording).

Safety

To ensure the safety of our eggs, all of the processes above were done with precision and care. Each well was transported with minimal shaking, and refreshing well solution or transporting individual eggs was done with minimal water pressure. To ensure personal safety, keep solutions away from sensitive body parts such as mouth or eyes to avoid potential dangerous side effects.

Results

Summary of Results

The experiment focused on determining potential effects of PTU on a developing embryo. The independent variable was the presence of PTU, which was 0% in one group, and 0.005% in the other group. The dependent variables, or variables possibly affected by PTU, measured were the survival rate of the fish, the percentage of fish that hatched, and the percentage that developed pigmentation. In order to ensure the comparison was an accurate cause and effect relationship, factors such as temperature, the amount of fluid in each well, the amount of shaking the fish were exposed to, and the length of time between solution renewals needed to be balanced out.

The experiment focused on the potential effects of PTU on developing zebrafish embryos. In this experiment, the independent variable that was focused on was PTU with a control group with 0% of the chemical in the water and 0.005% in the experimental group. Our hypothesis was supported as a result of our experiment. Throughout our experiment, the PTU exposed populations suffered from a higher mortality rate, a lower pigmentation percentage, and a lower percentage hatched. As the time passed post-fertilization increased, the difference between PTU exposed fish and control fish increased as well. While a majority of the control fish survived, developed pigmentation, and hatched, by Day 5 every PTU exposed fish had died, and none had developed pigmentation or hatched.

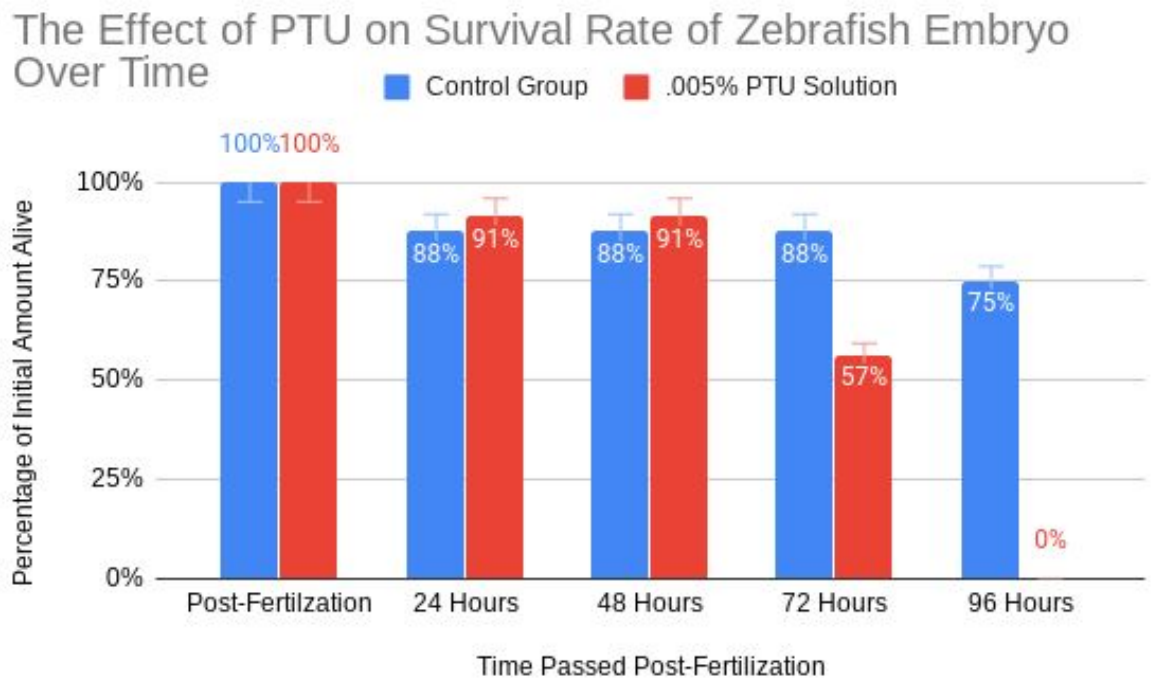
THE EFFECT OF PTU ON THE DEVELOPMENT OF ZEBRAFISH

Tables and Figures

Table 1: The Effect of PTU on the Percentage of Initial Zebrafish Alive (Survival Rate) Over Time

	Day 1 Post-Fertilization	Day 2 24 Hours	Day 3 48 Hours	Day 4 72 Hours	Day 5 96 Hours
Control Average	100%	87.5%	87.5%	87.5%	75%
Experimental Average	100%	91.3%	91.3%	56.5%	0%

Chart 1:



Survival Rate:

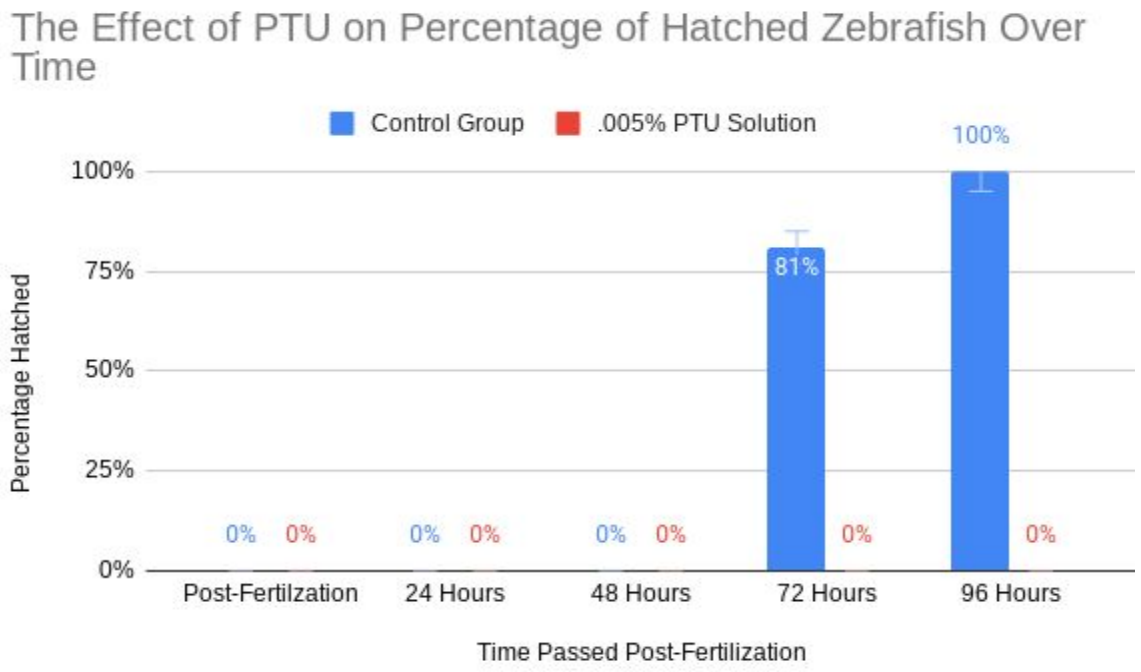
We calculated the survival rate as the ratio of how many were alive by the initial amount. Overtime, the survival rate of each population decreased; however, the survival rate of PTU exposed populations decreased more dramatically than the control group did in the last 48

hours of the experiment (as shown above in Chart 1). This supported our hypothesis, as we stated PTU exposure would have a negative effect on survival rate due to it potentially interfering with development, which our data suggests by a greater decline in survival rate for 0.005% PTU solution populations.

Table 2: The Effect of PTU on Percentage of Hatched Zebrafish Among the Living (Percentage Hatched) Over Time

	Day 1 Post-Fertilization	Day 2 24 Hours	Day 3 48 Hours	Day 4 72 Hours	Day 5 96 Hours
Control Average	0%	0%	0%	80.9%	100%
Experimental Average	0%	0%	0%	0%	0%

Chart 2:



Percentage of Hatched Zebrafish:

We calculated the percentage of hatched zebrafish using the ratio between the number of fish hatched and number of fish alive. Our data supports our hypothesis that PTU would decrease hatching percentage, as it suggests that PTU decreases the percentage of hatched zebrafish as shown by Chart 2 in the clearly larger percentages in the control group.

Table 3: The Effect of PTU on the Percentage of Pigmented Zebrafish Among the Living (Percentage Pigmented) Over Time

	Day 1 Post-Fertilization	Day 2 24 Hours	Day 3 48 Hours	Day 4 72 Hours	Day 5 96 Hours
Control Average	0%	0%	52.4%	95.2%	100%
Experimental Average	0%	0%	0%	0%	0%

Chart 3:

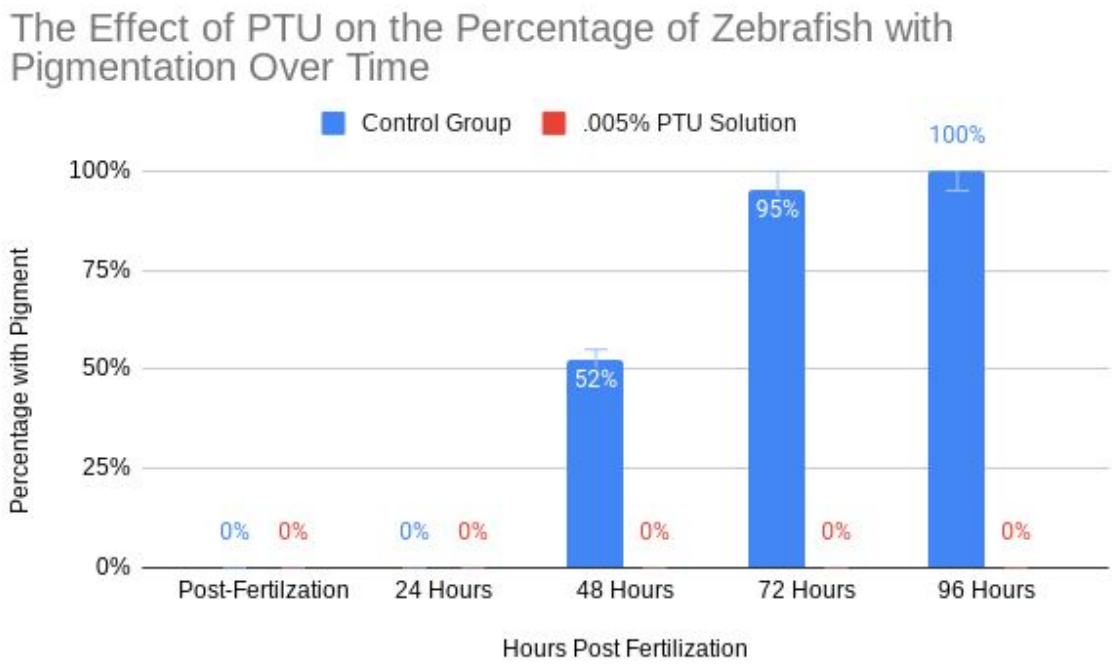
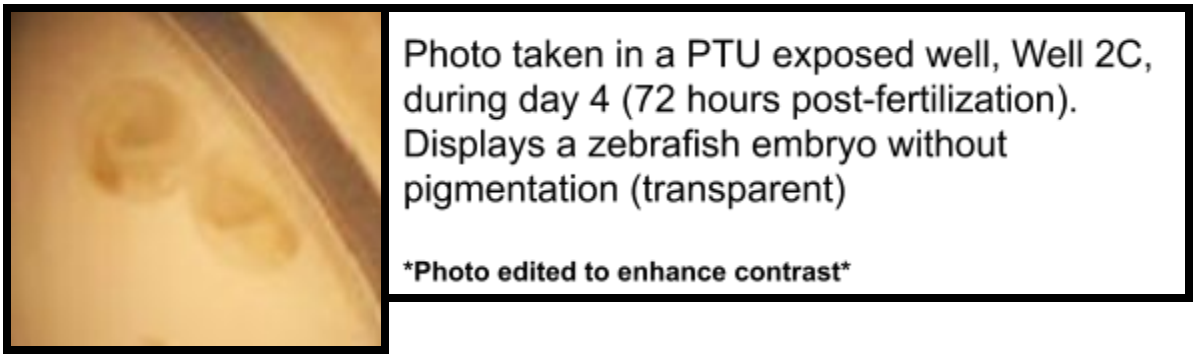


Figure 2: Pigmented Zebrafish in Control Solution

Figure 3: Zebrafish Lacking Pigmentation in PTU Treatment



Pigmentation:

Our data shows that the percentage of pigmented zebrafish decreases if a population is exposed to PTU, supporting our hypothesis. As seen in figure 2, nearly all fish in the control group had similar pigment. As seen in figure 3, all fish in the PTU treatments had similar transparency. Following our definition of pigmentation to make these observations quantifiable, we found that there was a significant difference in the percentage of zebrafish with pigment between the control group and PTU exposed group, as shown in Chart 3.

Statistical Significance

To determine the statistical significance of the data, an unpaired t-test was used. This test determines the likelihood that the results are a product of chance. An unpaired t-test determined that there was an extremely statistically significant difference in hatching percentage and pigmentation percentage between the two groups (p-value at 0.0001). Despite drastic differences in the end result for survival rates, it was not found as statistically significant (p-value at .1081) most likely due to a small sample size of only 6 populations. If there were trials performed in the future, the difference in survival rate would likely come out statistically significant if it followed the same trend as our data.

Discussion

Importance of the Topic

Understanding the potential effects of chemicals on developing embryos, whether it is zebrafish or human, is an incredibly complex and important issue. With as little as 0.005% of a PTU concentration, zebrafish have drastic effects ranging from a higher mortality rate to albinism. The decreased hatching rate suggests that the drug is a deadly toxin at even small exposures, and it helps us build a more complete idea of the fragility of a developing organism. Researching the effects of drugs on a developing zebrafish embryo could potentially lead to breakthroughs in discovering hidden reasons and solutions behind issues such as disorders or miscarriages. Knowing how the drug inhibits tyrosinase could expose a vulnerability in the enzyme, and could help us draw connections between other shortcomings in it's infrastructure. Having a full understanding of the functionality of tyrosinase would assist development of treatment for albinism that results in a mutation for the enzyme.

All present-day understandings of the dangers of toxins have been discovered through trial-and-error processes, much like this experiment; however, unfortunately, much of our knowledge in toxicology came at the cost of human experience, such as the human health issues discovered from accidental exposure to lead or mercury. In incidents such as the minamata incident, the catastrophic effects of exposure to mercury were revealed; however, it came at the cost of many people's lives and well being. If the damaging properties of toxins were determined prior to tragic incidents, the potential dangers could be limited to a lab setting without the eventual progression into human examples. By proactive measures and in depth research, we could potentially avoid many catastrophes that result from mass chemical exposure, while simultaneously discovering properties of drugs and qualities of humans.

Importance of the Findings

Finding a statistically significant link between PTU exposure decreasing pigmentation percentage and hatching percentage in zebrafish embryos can be very important for our understanding of PTU's potential effect on humans. Due to genetic similarities, it is very likely that humans would have a similar reaction to PTU as the negative one zebrafish had. The data from this experiment is potentially inaccurate, as we may have had a fungal infection killing off PTU infected zebrafish in greater amounts than control fish, since we had similar problems in nearby groups. Since scientifically accepted toxicology charts supported a much higher survival rate in our PTU exposed population than the one we achieved in our experiment, it is likely an outside factor had an impact on our results. Despite this, we still had significantly different data, and without that error we likely would have seen a similar trend. As a result of this experiment, we confirmed that PTU chemically inhibits tyrosinase, as well as having other unknown effects that prove to be fatal. Further research can reveal the vulnerabilities in embryonic development that negatively suffered from chemical exposure, as well as revealing the limitations to the enzyme tyrosinase. Both these discoveries can help us develop more thorough health procedures to protect future children as well as develop treatment for those who suffer from dysfunctional tyrosinase proteins.

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