The Effects of Hair Dye on the Development of Zebrafish Embryos

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Abstract

P-Phenylenediamine (PPD) is one of the many chemicals in hair dye and is a known toxin. Many women and men color their hair without giving a second thought to the dangers they may be exposing themselves to or their unborn child. There have not been enough studies to know how dangerous dyeing your hair during pregnancy is, especially the delicate first trimester. An experiment was conducted using Danio rerio (zebrafish) to see which ingredient, the developer; the color; or the mixture of the two, is most dangerous to humans. Zebrafish are suitable models because they are vertebrates like humans. Also, their clear embryos and fast rate of development allow research to be done effectively. This study exposed 30 zebrafish embryos to 200 µM of hair developer solution, 30 zebrafish embryos to 200 µM of hair color solution, and 31 zebrafish embryos to 200 µM of a mixture of developer and color to see the effects on the development. Those zebrafish that were exposed to hair dye were then compared to 30 zebrafish in the control group that were never exposed to hair dye. All of the embryos that were exposed to the color and the mixture of developer and color showed signs of deformities and low survival. The results of this experiment showed no signs that the developer would cause significant issues other than growth retardation. This experiment is significant to human health and shows that people should monitor what is in the hair dyes they use.

Introduction

Women in the United States have been dyeing their hair since Clairol instilled the fear of aging in their ad campaigns during the 1940s and 1950s. Gray hair, according to Clairol's copywriters, could be “the ruination of romance,” and leave a woman “buried beneath…dull, drab color" (Marshall, 2015). Hair dyes are used by more than one-third of women over 18 years old and about 10% of men over 40 in the United States and Europe (Hair Dyes and Cancer Risk, 2016). A wide range of people around the world use many hair dye products to change the color of their hair, but it seems no one really knows the extensive damage they could cause. The use of hair dyes can be traced all the way back to 4,000 B.C. (Manjunatha, Wei-bing, Ke-Chun, Marigoudar, Xi-qiang, Xi-min, and Xue, 2014).

Hair dyes consist of over 5,000 different chemicals, some of which are disclosed to be carcinogenic (cancer-causing) in animals. Scientists have tried to ascertain if exposure to the chemicals in hair coloring products increases a person’s cancer risk (Hair Dyes and Cancer Risk, 2016). Some hair dye formulas were found to cause cancer in animals which led manufacturers to change the components in dye products in the mid- to late 1970s. However, it is unknown if some of the chemicals in hair dyes that are used today can cause cancer. One of the chemicals in hair dye today is p-Phenylenediamine (PPD) and it is on the Hazardous Substance List. PPD can affect you when breathed in or by passing through the skin. A German study showed that PPD is the 5th most common skin allergen. When ingested PPD is highly toxic, causing respiratory distress and renal failure (P-Phenylenediamine). According to the National Cancer Institute, studies have shown an increased risk of certain cancers of the blood

and bone marrow, such as non-Hodgkin lymphoma (NHL) and leukemia, with the personal use of hair dyes. However, other studies have not shown the connection between hair dye and cancer. Conflicting results have also been produced in studies of breast and bladder cancer. There have been relatively few studies regarding the association of hair dye use with the risk of other cancers. The IARC Working Group stated that personal use of hair dyes is not classifiable as to its carcinogenicity to humans (Hair Dyes and Cancer Risk, 2016). Mayo Clinic's website reports that a 2005 study found an increased risk of the childhood cancer neuroblastoma in mothers that colored their hair during pregnancy. Although, other studies did not reach the same conclusion. There is no substantial proof that maternal use of hair dye before or during pregnancy increases the risk of childhood tumors (Hair dye and pregnancy: A concern?).

In the early 1970s Dr. George Streisinger, a scientist at the University of Oregon, discovered that zebrafish, Danio rerio, made an excellent model for studying vertebrate development and genetics because zebrafish eggs are clear and develop outside of the mother's body so scientists can watch a zebrafish embryo grow into a fish under a microscope (Zebrafish FAQs). Since zebrafish embryos, like humans, undergo a series of reproducible, identifiable stages during normal development and there are detailed drawings, images, and time-lapse videos describing the stages of zebrafish development, as well as morphological landmarks characteristic of the different developmental stages making them the perfect model. For example, the early stages of zebrafish development are characterized by rapid, synchronous cell divisions, so the effects of a chemical agent on this synchronization are noticeable, even to the casual observer. Also, defects in the development of the eyes or otic vesicles were readily noticeable (Tomasiewicz, Hesselbach, Carvan, Goldberg, Berg, and Petering, 2014). This is why zebrafish embryos were chosen for this experiment. The purpose of this experiment was to see how the hair dye developer, color, and mixture of developer and color affect the development of zebrafish embryos compared to the controlled group of unexposed embryos. Prior research in this field led to the following hypothesis: When zebrafish embryos are exposed to hair dye containing PPD, the embryos will display altered morphological and physiological abnormalities including increased mortality, hatching delay, or growth retardation.

Materials and Methods

Materials

- 1 container of 200 µM developer (Variable solution)
- 1 container of 200 µM color (Variable solution)
- 1 container of 200 µM mixture of developer and color (Variable solution)
- 1 bottle Instant Ocean/Embryo Media Solution (Control solution)
- Methylene Blue (Antiseptic solution)
- 120 zebrafish embryos
- 5 disposable pipettes (1.5mm for transferring and manipulating eggs)
- 2 disposable 1 mL pipettes (for each day testing)
- 1 waste beaker for dead embryos and liquid disposal
- Labeling tape

- Labeling marker (Sharpie)
- 1 3x4 falcon plate with 12 wells
- Microscope depression slide with coverslip
- Dissecting and compound microscopes
- 28.5℃ Incubator

Safety Precaution: Wear gloves when handling the materials.

Procedure

Day 1

1. Receive rinsed embryos from supervisor.
2. Use a sharpie marker to label your plate with your name and class hour. Label a row for the 200 µM developer, 200 µM color, 200 µM mixture and control.
3. Take one disposable pipette and fill the row labeled control of the well plate with 1 mL of Instant Ocean/Embryo Media solution.
4. Fill the remaining wells with a suitable amount developer, color or a mixture of developer and color.
5. Split the embryos up so that there are around 10 embryos in each well.
6. On the student data sheet, record the exact number of live embryos. Dispose of dead embryos.
7. Record observations of the embryos on student data sheet after your embryos have been observed under the dissecting microscope.
8. To prevent bacteria from destroying the embryos, add a drop of Methylene Blue.
9. Set the plate in the 28.5°C incubator overnight.

Day 3

1. Remove plate from incubator.
2. Use the disposable pipette to discard any of the dead embryos found. Squirt dead embryos into a waste beaker. Watch out for the alive embryos, be careful to only remove the dead ones.
3. Record the number of remaining embryos or hatched fish in the data table.
4. Remove hair dye solutions from each well of the plate using a pipette. Do this by removing the liquid from the top after tilting the plate so that the embryos settle.
5. Replace the developer, color and mixture with fresh developer, color, and mixture using a clean pipette every time.
6. Record observations of what you see when the the plate is set under the dissecting microscope on student data sheet. Take note of any developmental markers and abnormalities. Repeat for all hair dye combinations.
7. Take 1-2 embryos and set them on the depression slide with a coverslip. Record the observations you make while using the compound microscope in your student data sheet. Repeat for all hair dye combinations.
8. Place the embryos back in their well in the plate and add a drop of Methylene Blue In order to prevent bacteria from destroying the embryos.
9. Return the plate to the appropriate 28.5°C incubator

Day 3
1. Repeat day 2 work and observations. Record all data.
2. Record all observations in the data table.

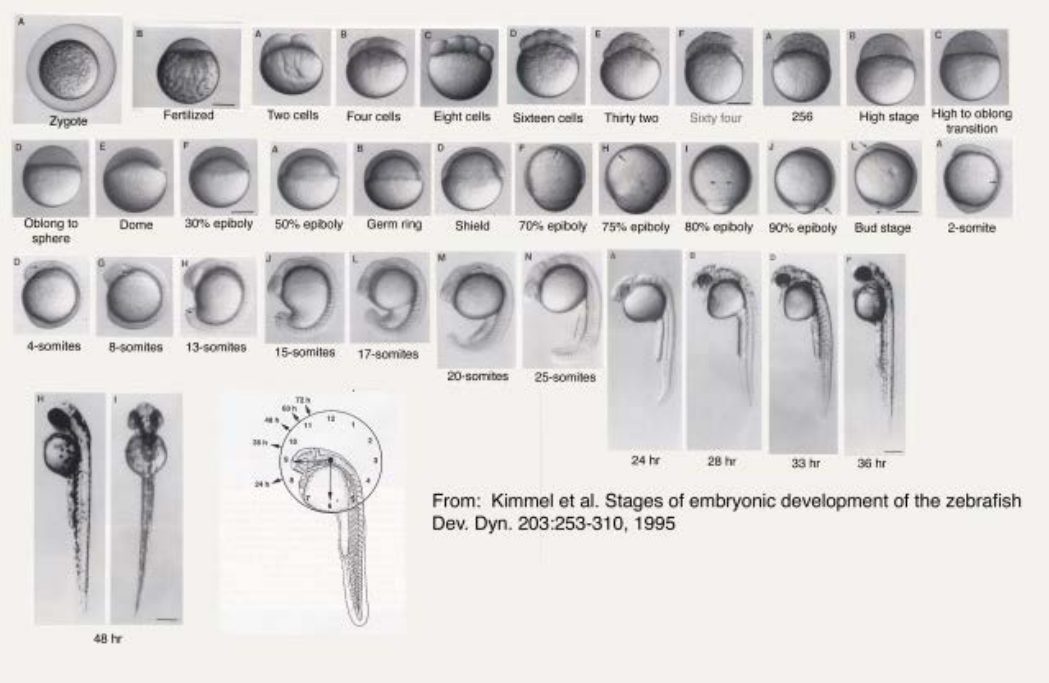
Day 4
1. Repeat the work done on day 2 and day 3.
2. Record all observations in the data table.
3. If day 4 is the last day of the experiment, see step 3 on day 5.

Day 5 (Optional)
1. Repeat day 2 and day 3 work and observations.
2. Record all observations in the data table.
3. Discard all remaining embryos and fish by placing them in waste container.
4. Clean up the rest of the materials used.

Procedure is from SEPA- UW-Milwaukee

Methods
A dissecting microscope and a compound microscope were used to count how many zebrafish embryos were still alive and how many had hatched as well as their development. To observe the fish without a microscope, the fish were placed over a piece of white paper. The following zebrafish development diagram (figures 1) was used to determine how the zebrafish embryos were developing. These outcomes were compared to the control group to find out how hair dye affects the embryos.

Figure 1



Data

Data Table 1
Observations of Zebrafish Embryos Exposed to Hair Dye Over a 72 hpf

Hair Dye Solution	Well No.	No. of Starting Embryos	48 Hours Post Fertilization			72 Hours Post Fertilization		
			# Dead	# Alive	# Hatched	# Dead	# Alive	# Hatched
Control	1	10	0	10	0	0	10	0
Control	2	10	0	10	0	0	10	0
Control	3	10	0	10	0	0	10	0
200 µM Developer	1	10	0	10	0	0	10	0
200 µM Developer	2	10	0	10	0	0	10	0
200 µM Developer	3	10	1	9	0	0	9	0
200 µM Color	1	10	3	7	0	0	7	0
200 µM Color	2	10	5	5	0	0	5	0
200 µM Color	3	10	7	3	0	0	3	0
200 µM Color & Developer	1	10	4	6	0	0	6	0
200 µM Color & Developer	2	10	5	5	0	0	5	0
200 µM Color & Developer	3	11	6	5	0	0	5	0

Data Table 2
Observations of Zebrafish Embryos Exposed to Hair Dye 96 hpf and 120 hpf

Hair Dye Solution	Well No.	No. of Starting Embryos	96 Hours Post Fertilization			120 Hours Post Fertilization		
			# Dead	# Alive	# Hatched	# Dead	# Alive	# Hatched
Control	1	10	2	8	8	0	8	8
Control	2	10	0	10	10	0	10	10
Control	3	10	1	9	9	0	9	9
200 µM Developer	1	10	0	10	8	2	8	8
200 µM Developer	2	10	2	8	8	0	8	8
200 µM Developer	3	10	0	9	9	0	9	9
200 µM Color	1	10	1	6	0	5	1	1
200 µM Color	2	10	1	4	1	0	4	2
200 µM Color	3	10	0	3	0	0	3	2
200 µM Color & Developer	1	10	3	3	0	3	0	0
200 µM Color & Developer	2	10	4	1	0	1	0	0
200 µM Color & Developer	3	11	5	0	0	0	0	0

Data table 1 shows how many zebrafish embryos were alive after a 48 hpf period and a 72 hpf period, and data table 2 shows how many zebrafish embryos were alive after a

96 hpf and a 120 hpf period. The zebrafish in the control group were in the instant ocean environment to be used to make comparisons between the variable groups. These fish were never exposed to hair dye and had a high survival rate of 90%. The zebrafish exposed to 200 µM of hair dye developer had a high survival rate of 83% hatched zebrafish. The zebrafish exposed to 200 µM of hair dye color had 15 die by 48 hpf and by 120 hpf 17% hatched and 73% were dead. The zebrafish exposed to 200 µM of mixture also had 15 die by 48 hpf. By 120 hpf all of the zebrafish embryos were dead.

Figure 2

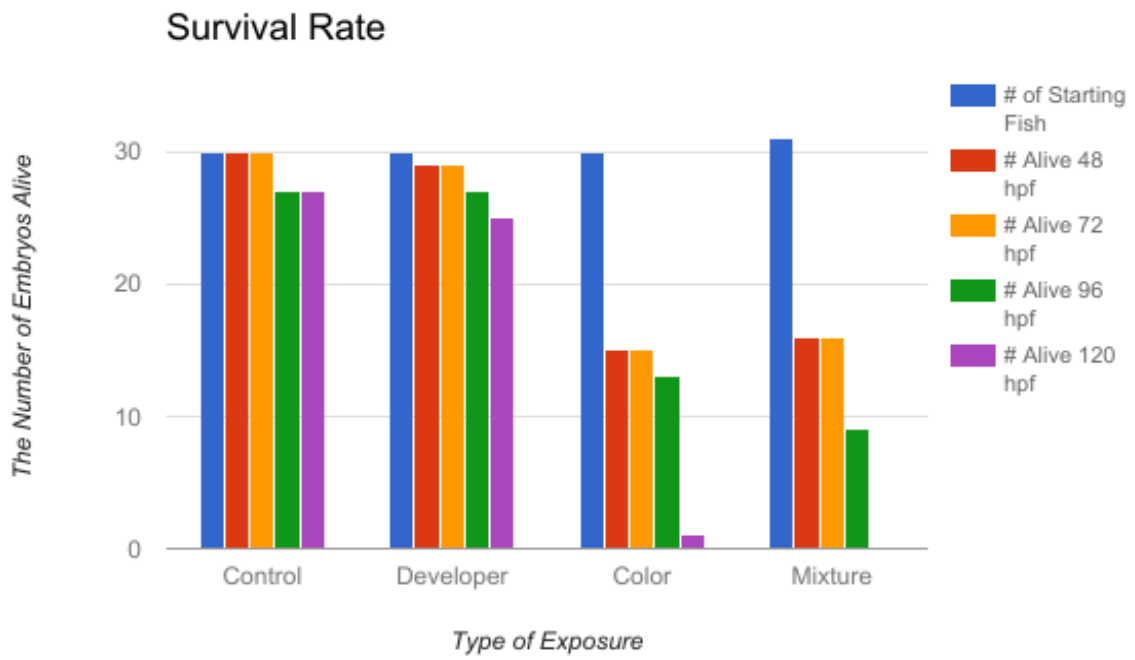


Figure 2 is a bar graph that shows visually how the survival rates of the zebrafish exposed to developer, color and a mixture of developer and color vary and compare to the control group.

Figure 3

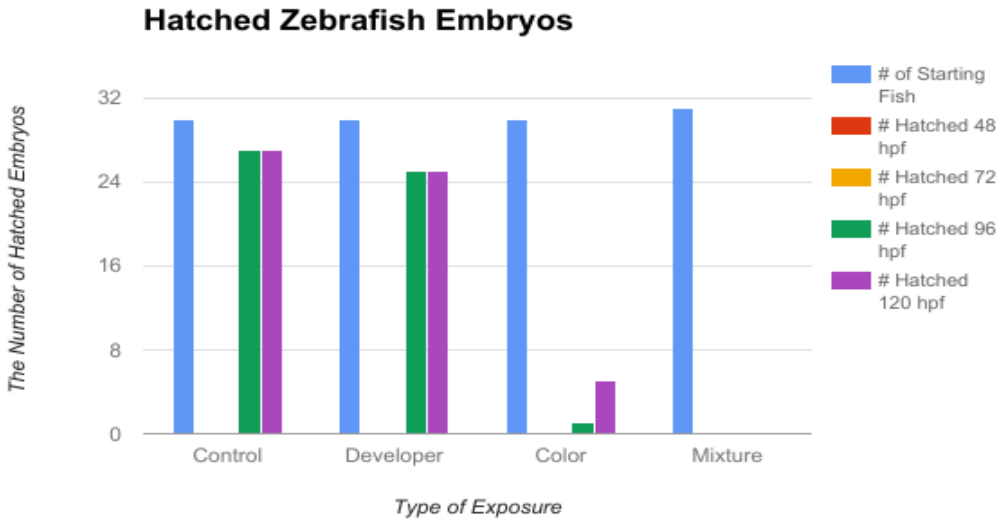


Figure 3 is a bar graph visually showing the number of zebrafish that hatched.

Figure 4

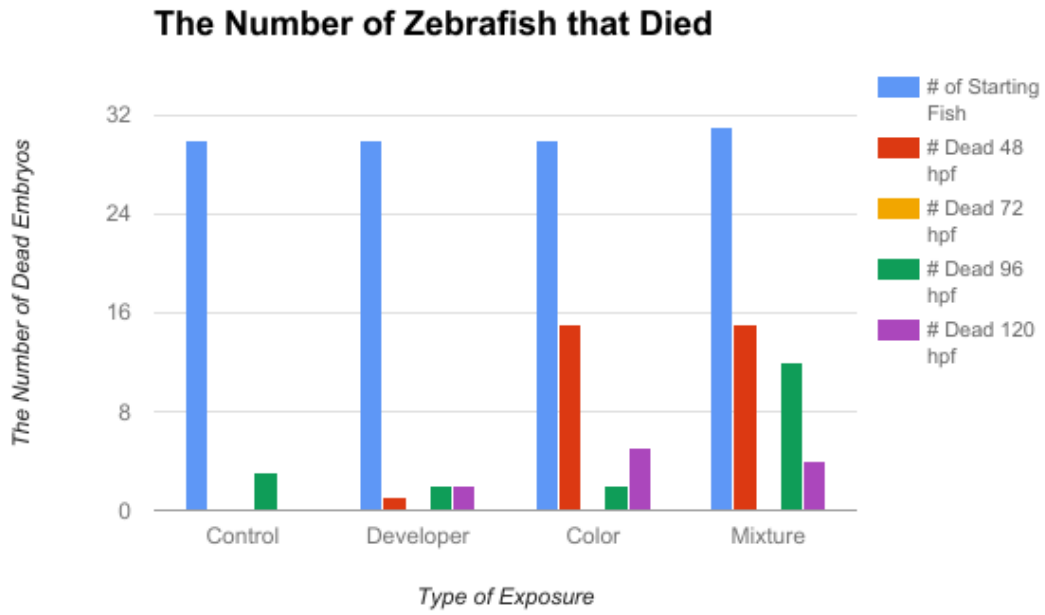


Figure 4 is a bar graph of the effects of hair dye on zebrafish embryos showing the number of zebrafish that died for each type of exposure to hair dye and the control group.

Figure 5

Photograph of zebrafish in the control group after 96 hours post fertilization showing normal development.

Figure 6

Photograph of a zebrafish exposed to the color solution after 96 hours post fertilization. You can see the deformity of this zebrafish with the curved tail.

Figure 7

Photograph of a zebrafish exposed to the developer solution after 96 hours post fertilization. The zebrafish embryo developed slower and is showing signs of deformity.

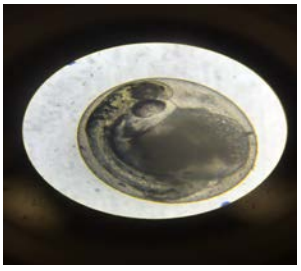


Figure 8

Photograph of a zebrafish exposed to both the color and developer solution after 4 days. This embryo was deformed and died.

Figure 9

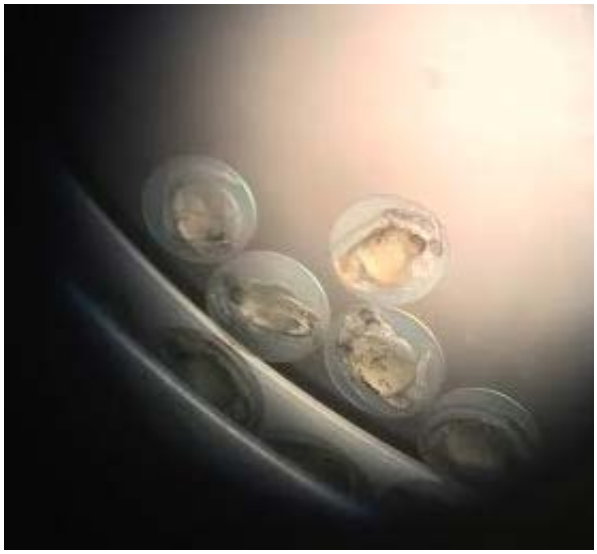


Figure 9 clearly shows the deformity of the embryos in the color group 96 hours post fertilization.

Data Analysis

An unpaired T-test is a statistical comparison of the means of two groups and is used to examine whether the two groups are in fact different by evaluating the differences in data and calculating the p-value. The p-value (alpha level) is the probability the difference found in the data is significant and not due to chance. A p-value of less than 0.05 is the goal level for this type of research and means that the probability of the difference seen in the data is due to random error in less than 5%.

Data Table 3

Number of Zebrafish Alive

Comparison	Day 2 P-value	Day 2 Statistical Significance	Day 3 P-value	Day 3 Statistical Significance
Control vs. Developer	.3739	Not Statistically Significant	.3739	Not Statistically Significant
Control vs. Color	.0123	Statistically Significant	.0123	Statistically Significant
Control vs. Mixture	.0002	Very Statistically Significant	.0002	Very Statistically Significant

Data Table 4

Number of Zebrafish Alive (continued)

Comparison	Day 4 P-value	Day 4 Statistical Significance	Day 5 P-value	Day 5 Statistical Significance
Control vs. Developer	1.0	Not Statistically Significant	.3739	Not Statistically Significant
Control vs. Color	.0114	Statistically Significant	.0039	Statistically Significant
Control vs. Mixture	.0019	Very Statistically Significant	.0001	Very Statistically Significant

Data tables 3 and 4 show the p-values found using the unpaired T-test comparing the control group to all three experimental groups after 48 hpf, 72 hpf, 96 hpf, and 120 hpf. The comparisons were made between the number of alive zebrafish from the control group and the number alive from the experimental groups exposed to 200 µM of developer, 200 µM of color, and 200 µM of a mixture of developer and color. The comparison between the control group and the developer were not statistically significant for any of the time periods. The comparison between the control group and the color group was statistically significant especially at 96 hpf and 120 hpf where they were very significant. The mixture compared to the control group was very statistically significant for all time periods.

Data Table 5

Number of Zebrafish Hatched

Comparison	Day 4 P-value	Day 4 Statistical Significance	Day 5 P-value	Day 5 Statistical Significance
Control vs. Developer	.3739	Not Statistically Significant	.3739	Not Statistically Significant
Control vs. Color	.0002	Very Statistically Significant	.0004	Very Statistically Significant
Control vs. Mixture	.0001	Very Statistically Significant	.0001	Very Statistically Significant

Data table 5 shows the p-values found using the unpaired T-test comparing the number of hatched zebrafish in the control group to the number of hatched zebrafish in all three experimental groups after 48 hpf, 72 hpf, 96 hpf, and 120 hpf. Again, the comparison between the control group and the developer were not statistically significant for any of the time periods. However, the comparison between the control group and the color

group and the control group and the mixture group were very statistically significant for all time periods. These results show the impact hair dye has on zebrafish embryos.

Results

In this experiment the control group was never exposed to hair dye. The control group was used to compare the experimental groups and lived in a variable free environment of the instant ocean solution. The independent variables in this study were the 200 µM concentration of developer, the 200 µM concentration of color, and the 200 µM concentration of the mixture of developer and color. The dependent variable was the number of deaths and abnormalities that resulted from the hair dye solutions. The constants used in this experiment were the one drop of methylene blue, the amounts of solution, the incubator temperature of 28.5°C, and the time of day the zebrafish embryos were exposed to the solutions. The constants make it possible to rule out outside variables that may effect the results and determine whether the hair dye solutions alone has an effect or not.

On day one all embryos were examined and determined to be healthy and fertilized. Ten healthy embryos were placed in each well. The zebrafish embryos were later examined at the 48 hours post fertilization (hpf) period. Out of the 30 embryos in the control group, all of the embryos were developing at a healthy rate and the spines were starting to form. In the group that contained the developer 29 out of 30 embryos had survived. Their survival was indicated by some quick twitching movement in the chorion. These zebrafish embryos appeared to be developing normally. Half of the 30 embryos exposed to the color died. The embryos that were alive closely resembled the embryos in the control group. The embryos in the mixture contained 16 dead embryos out of the 31 embryos. The remaining embryos seemed to be slightly lagging the development of the control group.

The zebrafish were placed into the incubator overnight and after 72 hpf the embryos were examined again. The control group continued to show normal development. The embryos all had normal body parts, normal heart beats, and did not have the slight bend in the tail like the embryos that were exposed to the hair dye. In the developer group there were also no deaths. No more embryos died in the color group, but the embryos appeared to have a slow rate of development. The tails of the embryos were still bundled up inside of the chorion. Again, none of the embryos died in the mixture group; however, the embryos continue to show a lag in development. The embryos eyes were clear. Compared to the control group the tails were undersized and their stomachs were enlarged.

Observations were obtained 96 hpf. In the control group there were three deaths and 27 hatched. Their heartbeats were steady and normal and all body parts were at a normal proportion. These zebrafish looked very different compared to the embryos exposed to hair dye. Two of the embryos exposed to the developer died and 25 hatched; however, the hatched zebrafish were smaller than the control group. The embryos exposed to color contained 2 dead and 14 alive including 1 that hatched, although the zebrafish had a curved tail. The 13 embryos continued to lag in development and looked deformed. Twelve of the embryos exposed to the mixture died and the 4 living embryos were deformed with abnormally inflamed stomachs. When testing the reaction with a minimum bore 1.5 mm disposable pipette, the zebrafish in the

color and the mixture wells reacted slowly compared to the control group or did not move at all when touched.

At 120 hpf, the 27 zebrafish in the control group seemed to be at the correct developmental stage with only 1 appearing smaller. The 25 zebrafish in the group exposed to the developer continue to thrive; however, they are still small than the control group. The final 2 embryos died in the developer group. Four of the embryos in the color group hatched. The fish that hatched had curved spines resulting in very bent tails. All of the zebrafish embryos exposed to the mixture were dead.

The aim of the experiment was to find out the effects of hair dye on zebrafish embryos and which part of the dying process was more damaging to the development of the zebrafish. The quantitative data found by the death rate was significant between the embryos exposed to the color and the mixture. The qualitative data recorded showed that zebrafish embryos exposed to hair dye developer, color and a mixture of developer and color causes deformities and can even cause death. The data showed that the color and the mixture of color and developer were highly damaging to the embryos.

Discussion

There was plenty of significant qualitative data recorded in this experiment suggesting that hair dye had an impact on the zebrafish embryos. The color which contains PPD and the mixture of color and developer affected the embryos the most. After 48 hpf half of the embryos in these two solution died and the other half showed delayed growth and developmental deformities. This data supports the hypothesis that when zebrafish embryos are exposed to hair dye containing PPD, the embryos will display altered morphological and physiological abnormalities including increased mortality, hatching delay, or growth retardation. The developer which does not contain PPD did not have a major impact on the zebrafish embryos. However, the 25 hatched embryos that were exposed to the developer did display growth retardation. A possible source of error could be that the embryo was deformed to begin with before it was even exposed to the hair dye. Further experiments could be done exposing the zebrafish embryos to the hair dye on different days to see if it makes a difference on when they are exposed. Therefore, it would give doctors a better idea if women should avoid using permanent hair dye during the first trimester or throughout the entire pregnancy.

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