The Effects of Hair Dye on the Development of Zebrafish Embryos

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Abstract:

Dyeing hair is a routine part of many women's lives. However, pregnant women may need to start taking precautions when it comes to dying their hair. The exposure to hair dye containing P-Phenylenediamine (PPD) could have an impact on a fetus in the womb. Zebrafish were used to see whether or not hair dye containing PPD could have an impact on a developing organism. For five days 30 zebrafish embryos were exposed to 1 mL of 200µM developer, 30 zebrafish embryos were exposed to one mL of 200µM color, 31 zebrafish embryos were exposed to one mL of 200µM mixture of color and developer, and 30 zebrafish embryos were exposed to one mL of a solution of instant ocean as a control group. The results of the study concluded that the zebrafish embryos exposed to 200µM color and 200µM mixture of developer and color had an increased death rate, curvature of the body, slow or nonexistent reaction, enlarged pericardial sac, smaller bodies, and in some cases no tail. Hair dye has become an essential in many lives of women, but while pregnant women need to be careful about exposing the fetuses they are carrying to hair dye containing PPD, as it may lead to harm to the fetus. This study gives reasonable doubt on using hair dye while pregnant and opens up the suggestion of more research into how hair dye can affect a fetus in the womb.

Introduction:

P-Phenylenediamine (PPD) is a chemical used in more than two-thirds of permanent hair dyes. When mixed with an oxidizing agent, which strips the color out of the melanin, PPD causes hair to turn shades of brown. In order to control what color the hair becomes, coupling agents are added to the mixture in order to react with previous ingredients to produce a specific color. While hair dye companies claim that the coupling agents desensitize the PPD, causing no allergic reactions to occur to the body, complaints from consumers and tests on animals have proved otherwise. (Brunning, 2015)

A study done on mice put a popular brand hair dye on the dorsum of the mouse ear. The mice experienced significant skin inflammation and cell infiltration on the first exposure. As the mice were exposed again and again the inflammation only increased minimally. On the fourth exposure the mice hit a peak point of inflammation with a thickness of 130-140% of the mice in the control group. The study showed that immune system can control the inflammation, but only after repeated exposure. (Rubin, et al, 2010)

To further the previous study done, a new study was done focusing separately on the color gel, developer, and a mixture of the two on mice. As a mixture, which is how the hair dye would be applied to humans, 20% more inflammation occurred more than the oxidizer and color gel alone. (Bonefeld, et al, 2009)

Henna is often used in hair dyes in Asian countries. In an experiment with zebrafish, the fish were exposed to different concentrations of henna hair dye. The hair dye caused many abnormalities, such as death, delayed hatching, tail deformation, and a weak heartbeat. (Manjunatha, et al, 2014)

In order to test the effects that hair dye containing PPD could have to a fetus in the womb, zebrafish will be used to demonstrate the effects. Zebrafish are often used to demonstrate the effects toxins have because of the parallels the zebrafish embryos and human fetuses have. Zebrafish help us to see possible abnormalities in development by developing outside the mother in a clear egg. Along with being able to see easily, the zebrafish develop fast so the effects of toxins can be seen in a week or less. (Petering, et al, 2010)

This study is looking at the effect hair dye has on zebrafish embryos. It is hypothesized that by exposing zebrafish to hair dye containing PPD, zebrafish embryos will have abnormalities in their physiological development.

Materials and Methods:

Acknowledgment: The procedure was taken from SEPA-UW-Milwaukee.

Warning: Use gloves while completing the following experiment.

Materials and Equipment:

- 1 container of 200µM developer
- 1 container of 200µM color
- 1 container of 200µM mixture of developer and color
- Beaker for dead embryos and liquid disposal
- Sharpie
- Tape
- Instant Ocean (Control solution)
- 1 Disposable pipette, minimum bore, 1.5 mm for transferring eggs to observation container and manipulating them in container
- 5 Disposable pipettes, 1 mL (for each day of testing)
- 1 3X4 Well Plate
- 28.5℃ Incubator
- Depression slide with cover slip
- Dissecting and compound microscope
- 120 Zebrafish Embryos
- Methylene Blue
- Data Table

Procedure:

Day 1:

1. Acquire the embryos, rinsed, from the teacher.
2. Using the sharpie and tape, label the well plate with the name of student(s) completing the experiment and the class hour.
3. Using the sharpie and tape again, label a row for the 200µM developer, 200µM color, 200µM mixture, and control group.

4. Fill the row labeled control group of the well plate with 1 mL of Instant Ocean/Embryo Media solution in each well using the disposable pipette.
5. Fill the remaining rows of the well plate with either developer, color, or a mixture of the two, using a new pipette each time.
6. Put about 10 embryos in each well.
7. Record the exact number of live embryos.
8. Remove any dead embryos from the well plate and discard properly. ***Make sure only dead embryos are removed.***
9. Observe the embryos in the well plate under the dissecting microscope, and write any observations in development in the data table.
10. Add a drop of Methylene Blue in order to prevent bacteria from destroying the embryos.
11. Place the well plate in the 28.5℃ incubator overnight.
12. Dispose of the pipettes used.

Day 2:

1. Retrieve the well plate from the incubator.
2. Using the disposable pipette, remove any dead embryos, and dispose of them into the waste beaker. ***Make sure only dead embryos are removed.***
3. Count the remaining embryos and the number of embryos that have hatched in each well. Record the statistics in the data table.
4. Remove the old developer, color, or mixture from the well plate by tipping the plate so the embryos settle, and remove the liquid from the top.
5. Using a new disposable pipette for each, replace the developer, color, and mixture with fresh developer, color, and mixture.
6. Record the observations of the embryos on the data sheet by placing the well plate underneath the dissecting microscope. Make sure to note any abnormalities in development. Repeat for all combinations of hair dye.
7. Place 1-2 embryos from the well plate on the depression slide with the cover slip. Observe the embryos using the compound microscope, and record any abnormalities in development and other observations in the data table. Repeat the previous for all combinations of hair dye.
8. Return the embryos to the appropriate well in the well plate.
9. Add a drop of Methylene Blue in order to prevent bacteria from destroying the embryos.
10. Place the well plate in the 28.5℃ incubator overnight.
11. Dispose of the pipettes used.

Day 3:

1. Repeat the work done on day 2.
2. Record all of the data observed in the data table.

Day 4 :

1. Repeat the work done on day 2 and day 3.
2. Record all of the data observed 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 the work done on day 2, day 3, and day 4.
2. Record all the data observed in the data table.
3. Dispose of all the remaining fish and embryos by placing them in the waste container.
4. Clean up or dispose of the rest of the materials properly.

Hair Dye Effect on Zebrafish (Data Table One)

Hair Dye Solution	Well #	# of Starting Embryos	Day 2			Day 3			Day 4			Day 5		
			# Living	# Dead	# Of Fish	# Living	# Dead	# Of Fish	# Living	# Dead	# Of Fish	# Living	# Dead	# Of Fish
Control	1	10	10	0	0	10	0	0	8	2	8	8	0	8
	2	10	10	0	0	10	0	0	10	0	10	10	0	10
	3	10	10	0	0	10	0	0	9	1	9	9	0	9
200 µM Developer	1	10	10	0	0	10	0	0	10	0	8	8	2	8
	2	10	10	0	0	10	0	0	8	2	8	8	0	8
	3	10	9	1	0	9	0	0	9	0	9	9	0	9
200 µM Color	1	10	7	3	0	7	0	0	6	1	0	1	5	1
	2	10	5	5	0	5	0	0	4	1	1	4	0	2
	3	10	3	7	0	3	0	0	3	0	0	3	0	2
200 µM Color & Developer	1	10	6	4	0	6	0	0	3	3	0	0	3	0
	2	10	5	5	0	5	0	0	1	4	0	0	1	0
	3	11	5	6	0	5	0	0	0	5	0	0	0	0

Observations:

Day 1:

When given all embryos were fertilized, but had not developed any further yet.

Day 2:

In the control group, the spines were starting to form.
In the color group, three embryos died, but were still as developed as the others. They were recently dead.

Day 3:

The fish in all of the experimental groups are not developing as well as the others.

Day 4:
In the developer wells, the fish that had hatched were smaller than the control.
The fish in the color and mixture group, could not have size compared, due to only one being hatched.

Day 5:

In the control group, one zebrafish was a smaller, but the rest seemed to be at the correct developmental stage.
In the color and mixture groups, the zebrafish were small, as noted in the developer group on day four, as well as the spine of the zebrafish was extremely curved.
Reaction was tested by lightly touching the fish with a minimum bore, 1.5 mm, disposable pipette. The zebrafish in the color and mixture wells, reacted slowly, compared to the control group, or did not move at all when touched.

Data table one shows the amount of fish alive, dead, or hatched on any given day in each of the solutions. The data table also notes any qualitative observations.

Number of Zebrafish Alive (Data Table 2)

Comparison	Day 2 P-value	Day 3 P-value	Day 4 P-value	Day 5 P-value
Control vs. Developer	.3739 (insignificant)	.3739 (insignificant)	1.0 (insignificant)	.3739 (insignificant)
Control vs. Color	.0123 (significant)	.0123 (significant)	.0114 (significant)	.0039 (significant)
Control vs. Mixture	.0002 (significant)	.0002 (significant)	.0019 (significant)	.0001 (significant)

Data table two shows the P-values of an unpaired t-test on the number of zebrafish alive on each day in each experimental group compared to the control group.

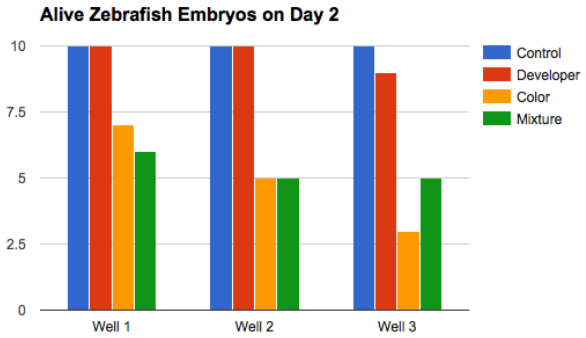
Number of Zebrafish Hatched (Data Table Three)

Comparison	Day 4 P-value	Day 5 P-value
Control vs. Developer	.3739 (nonsignificant)	.3739 (nonsignificant)

Control vs. Color	.0002 (significant)	.0004 (significant)
Control vs. Mixture	.0001 (significant)	.0001 (significant)

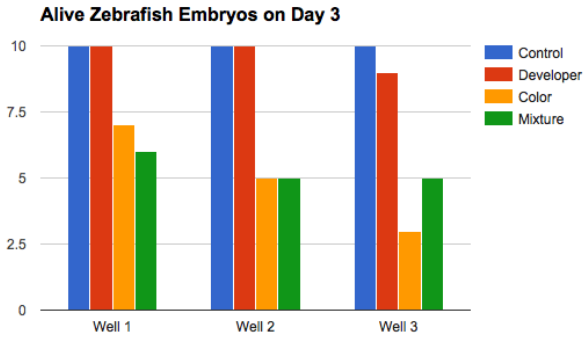
Data table three shows the P-values of an unpaired t-test on the number of zebrafish hatched on each day in each experimental group compared to the control group.

Graph One

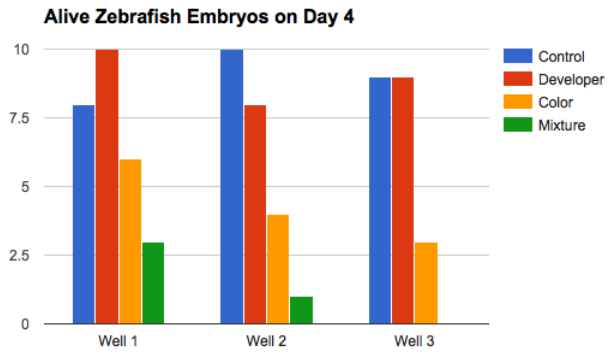


Graph one shows a comparison of the amount of zebrafish embryos alive on day two in each well in each solution.

Graph Two



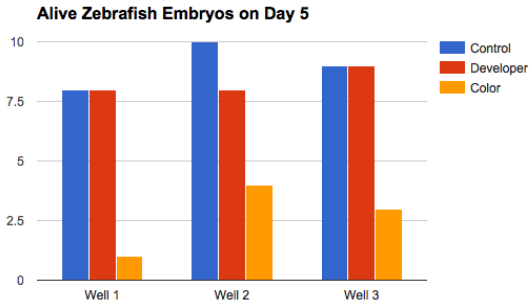
Graph two shows a comparison of the amount of zebrafish embryos alive on day three in each well in each solution.



Graph Three

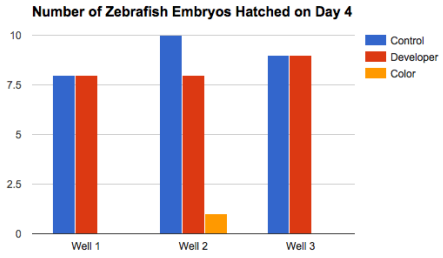
Graph three shows a comparison of the amount of zebrafish embryos alive on day four in each well in each solution.

Graph Four



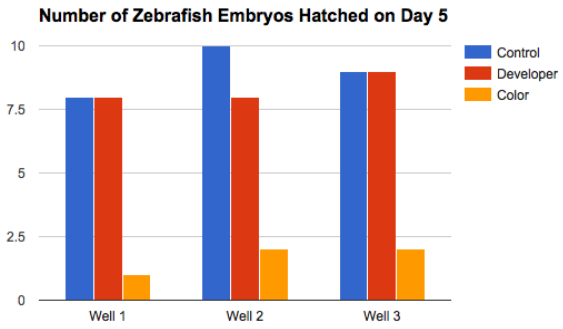
Graph four shows a comparison of the amount of zebrafish embryos alive on day five in each well in each solution.

Graph Five



Graph five shows a comparison of the amount of zebrafish embryos hatched on day four in each well in each solution.

Graph Six



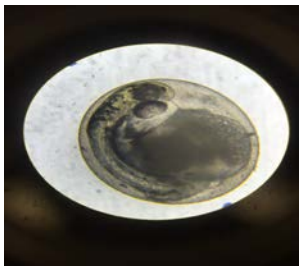
Graph six shows a comparison of the amount of zebrafish embryos hatched on day five in each well in each solution.

Image One: Two zebrafish in the control group on day four.

Image Two: A zebrafish in the color group on day four.

Image Three: A zebrafish embryo in the developer group on day four.

Image Four: A zebrafish embryo in the mixture group on day four.



Data Analysis:

In order to show whether or not there was a significance in death and hatching rates of zebrafish embryos exposed to 200µM hair dye developer, 200µM color of hair dye, and 200 µM mixture of hair dye developer and color compared to the zebrafish embryos who were not exposed, an unpaired T-test was used. If the P-value that resulted from the unpaired T-test was less than .05 the data is significant. Data table two shows the P-values of the control group versus each experimental group, and whether or not the P-value signifies an increase in the death rate of zebrafish embryos on each given day. On all days of the experiment, the developer did not show an increase in the death rate. However the 200µM color group and the 200µM mixture groups both had a significant P-value. These results indicate the 200µM color, which is in the mixture, caused an increase in the deaths of zebrafish embryos. Data table three shows the P-values of the control versus each experimental group, on days four and five, and whether or not the results were significant. Days four and five are the only days being compared because they were the only days in which hatching occurred in any group. Data table one shows the amount of zebrafish embryos alive, dead, and hatched on each day in the control group and each experimental group. Graph one shows the amount of zebrafish embryos alive on day two in each well in the control group and the experimental groups. Graph two shows the amount of zebrafish embryos alive on day three in each well in all groups. Graph three shows the amount of zebrafish embryos alive on day four in each well in all groups. Graph four shows the amount of zebrafish embryos alive on day five in each well in all groups. Graph five shows the amount of zebrafish that hatched on day four in each well in the control group, 200µM developer group, and 200µM color group. The 200 µM mixture of developer and color is not included on the graph because no embryos hatched on day four. Graph six shows the amount

of zebrafish that hatched on day five in each well in the control group, 200µM developer group, and the 200µM color group. Once again, the 200µM mixture of developer and color was not included in the graph because none of the zebrafish embryos hatched. Figure one shows two zebrafish in the control group on day four with no developmental issues. Figure two shows a zebrafish in the 200µM color group on day four. The zebrafish has a curved body with an enlarged pericardial sac. Figure three shows a zebrafish in the 200µM developer group on day four. The zebrafish has an enlarged pericardial sac and no tail. Figure four shows a zebrafish in the 200mM mixture of color and developer group on day four. The zebrafish did not hatch and was dead.

Results:

In this experiment zebrafish embryos were exposed to 200µM color, 200µM developer, and 200µM mixture of color and developer over a course of five days to see if the zebrafish embryos survived, died, or had developmental issues. A control group of zebrafish embryos was also observed in order to compare the data. The independent variables in this experiment were the different solutions the zebrafish were exposed to: 200µM color, 200µM developer, and 200µM mixture of developer and color of hair dye. The dependent variables was the amount of zebrafish embryos alive each day. The control factors of the experiment on how hair dye affects zebrafish embryos were the incubator temperature, methylene blue, instant ocean, the amount of solution, the concentration of the solution, and the time of day the fish were re-exposed to the solutions. All of the zebrafish embryos started off fertilized and with no developmental issues. The control group and each experimental group was given three wells. In each well ten zebrafish embryos were placed, with an exception of eleven zebrafish embryos being placed in the third well of the 200µM mixture of color and developer group. On day two, no zebrafish embryos had died in the control group, one zebrafish embryo died in the 200µM developer group, fifteen zebrafish embryos died in the 200µM color group, and fifteen zebrafish embryos died in the 200µM mixture of developer and color group. The zebrafish that died seemed to still have been as developed as the alive zebrafish, indicating that the zebrafish developed then died. On day three, no zebrafish embryos died in any well, but the zebrafish in the experimental groups were not as developed as those in the control group. On day four, three zebrafish embryos died in the control group, two zebrafish embryos died in the 200µm developer group, two zebrafish embryos died in the 200µM color group, and seven zebrafish embryos died in the 200µM mixture of developer and color group. On day four, the zebrafish embryos also started to hatch. 27 zebrafish embryos hatched in the control group, 25 zebrafish embryos hatched in the 200µM developer group, one zebrafish embryo hatched in the 200µM color group, and no zebrafish embryos hatched in the 200µM mixture of color and developer group. The fish that hatched in the 200µM developer group are smaller than the zebrafish hatched in the control group. Due to only one zebrafish being hatched, the 200µM color and 200µM mixture of developer and color could not have the size of hatched zebrafish compared. On day five, no zebrafish died in the control group, two zebrafish died in the 200µM developer group, five zebrafish died in the 200µM color group, and four zebrafish died in the 200µM mixture of developer and color group. Also on day five, no more zebrafish hatched in the control group, no more zebrafish hatched in the 200 µM developer group, four zebrafish hatched in the 200µM

color group, and no zebrafish hatched in the 200µM color and developer group. In the control group one hatched zebrafish was smaller than the others, but all other zebrafish in the control group were properly developed. In the 200µM color and 200µM mixture of developer and color, the zebrafish were small and curved, like observed in the 200µM developer group on day four. Reaction was tested on all of the groups by lightly touching the zebrafish with a minimum bore, 1.5 mm, disposable pipette. The 200µM color group and the 200µM mixture of color and developer group reacted much slower, or did react at all, than the control group when touched. The 200µM developer group reacted similarly to the control group when touched. In conclusion to the experiment, 27 zebrafish were alive in the control group, 25 zebrafish were alive in the 200µM developer group, eight zebrafish were alive in the 200µM color group, and no zebrafish lived in the 200µM developer and color group. Out of the remaining fish, all were hatched except for three zebrafish embryos in the 200µM color group. The data showed that 200µM color and 200µM mixture of color and developer can increase the death rate of zebrafish embryos and lead to developmental issues involving small size, curvature of the body, and a slow or nonexistent reaction. The 200µM developer group, while developed at a slower rate, did not have any long term developmental issues, nor have an increase in death rate that was significant, according to an unpaired t-test.

Discussion:

The experiment suggests that the hypothesis was two-thirds correct. Exposing the zebrafish embryos to hair dye containing PPD caused physiological deformities in the zebrafish embryos in two of the three experimental solutions the zebrafish embryos were exposed to: the 200µM color group and the 200µM mixture of color and developer. The zebrafish in the 200µM color group and the 200µM mixture of color and developer experienced an increased death rate, curved bodies, enlarged pericardial sacs, small bodies, slow or nonexistent reaction rate, and some zebrafish with no tails. A qualitative observation was noticed within the first two days that 200µM developer group did have a delay in development, but the development was soon at the same point as the control group and no statistical significance was found. Because of the color being in both the 200µM color group and the 200µM mixture of color and developer, it is reasonable to believe the color is the part of the hair dye which affected the zebrafish embryos. Due to limited resources and abilities at the high school level, the experiment was restricted in finding whether it was the PPD that causes the developmental issues and the increased death rate. Hair dye containing PPD however did cause developmental issues and an increased death rate in the zebrafish embryos. The experiment concludes that using hair dye containing PPD, while pregnant could cause harm to the fetus. More in depth research would help to see exactly what part of the color of hair dye affects the zebrafish embryos, whether it is PPD or not.

References:

Dabelsteen, S. (2010). Repeated Exposure to Hair Dye Induces Regulatory T-Cells in Mice**.** *British Journal of Dermatology*, 2010, 992-998.

Larson, J.M. (2009). Consumer Available Permanent Hair Dye Products Cause Major Allergic Immune Activation in an Animal Module. *British Journal of Dermatology*, 2009,

102-107.

Petering, D. (2014). Zebrafish as Models: Studying the Effects of Environmental Agents on Human Health. *Zebrafish*, 2014, 1-96.

Xue, W. (2014). The Effects of Henna (Hair Dye) on the Embryonic Development of Zebrafish. *Environ Sci Pollut Res*, 2014, 7-14.