The Effects of Varying Concentrations of Salicylic Acid on the Survival Rate of Zebrafish Embryos by Natalie Maufort

Abstract

Over the course of five days fertilized zebrafish embryos were observed after being exposed to varying salicylic acid levels on the first day. The concentrations were 2%, 4%, 6%, 0.02%, 0.04%, 0.0002%, 0.0004%, based on the percentage of salicylic acid in beauty cleansers and possible environmental exposure. As it had been hypothesized, increasing salicylic acid levels increased death rates in the zebrafish embryos. All concentrations were found to be very significant, in that it killed all fish in the wells. After several dilutions the fish sustained life in the 0.0002% 0.0004% concentrations, showing no significance. This data was alarming in comparison to other data, as it had shown that up to 4% salicylic acid was harmless..

With salicylic acid being so common in everyday routines it is important to understand its effects on the body. The salicylic acid showed drastic negative health effects on the zebrafish embryos. With the development of the zebrafish and humans being so similar it can be inferred that there should be caution with the use of salicylic acid during pregnancy.

Introduction

As of recent the beauty world has exploded, and with that came the desire for porcelain clear skin. Perfect skin can be achieved in a variety of ways, with a wide range of, sometimes dangerous, chemicals. Today many cleansers contain the chemical Salicylic acid. Originating from the bark of a Willow tree Salicylic acid is a beta hydroxy acid, making it oil soluble, a very important quality when it comes to a face wash, for it penetrates pores deeper than a water soluble ingredient would. As to what it actually does as a cleanser, it breaks down bacteria in the skin and acts as an exfoliant, relieving irritated blemishes. This being said, its over usage can lead to dry and irritable skin or even salicylate poisoning. Those who are pregnant or on blood thinners are discouraged to use products that include it (Jacques, 2019).

In studies it has shown that salicylic acid does not affect zebrafish embryos in terms of growth rate and histological changes, and they were actually quite insensitive to it, however, the

concentrations that had been tested were far less than what some face washes contain (Zivna, 2016). As with anything that is applied to the skin during pregnancy, it does enter the bloodstream, ultimately reaching the baby, so there is reason for caution. While it has not been tested in zebrafish, studies do show that in late term pregnancies, the use of high doses of salicylic acid increases the risk of intracranial bleeding in the fetus (Fairhall, 2009). Very few skin cleanser ingredients have been tested in pregnancy, so overall, skin care as a whole is to be weary of (Gibson, 2017). It has been suggested that if the products being used contain salicylic acid while pregnant, it should be no more than 2% (Barrell, 2018).

Why zebrafish? The research on zebrafish has exploded in the last few years due to the fact the fish are the perfect test subject. The fish are very easy and inexpensive to maintain (Parngwen, 2002). The gestation period of the zebrafish is significantly shorter than those of other test subjects. Due to the zebrafish's size it is easy to house. The fish develop outside of the mother, making it easy to see development and causing no hardship to the mother. The development of a zebrafish in the early stages is very similar to that of a human, thus toxicants affect them in similar ways (Why use zebrafish in research?, 2014). This experiment is looking at how salicylic acid impacts the development of zebrafish embryos, and if there is any concern with its usage in human development. With increasing salicylic acid concentrations there is reason to believe the survival rate of the zebrafish embryos will decrease.

Materials and methods

Quantity	Item
1 bottle per group	Stock solution of Salicylic Acid (2%, 4%, 6%, 0.02%, 0.04%, 0.0002%, 0.0004% salicylic acid)
1 per group	Beaker for dead embryo and liquid disposal
1	Sharpie
1 bottle per group	Instant Ocean
1 per group plus extras	Disposable pipette (1.5 mm)

1 per group	Disposable pipette (1 mL)
1 per group	Plate with wells
1	28.5℃ Incubator
1 per group	Depression slide with cover slip
1 per group	Dissecting and compound microscope

Day 1

A. Obtain rinsed embryos from the teacher,
B. Label the well plate. Label the salicylic acid concentration of each well using the sharpie provided
C. Fill the one well of the play with 1 mL of Instant Ocean/Embryo Media solution using the disposable pipette. Fill the remaining well with the appropriate salicylic stock solutions. Divide the embryos so there are approximately 10 embryos in each well. Label the plate on the student data sheet.
D. Record exact numbers of live embryos on student data sheet.
Note: Dead embryos should be discarded.
E. Observe the embryos under the dissecting microscope. *Record observations on student data sheet*
F. Place each plate in the 28.5°C incubator

Day 2

A. Remove plate from incubator.
B. Remove dead embryos from the plate using the disposable pipette. Squirt dead embryos into waste beaker. Be careful to only remove the dead embryos
C. Count remaining embryos, hatched fish, and *record in data table*
D. Remove salicylic acid stock solutions from each well of the plate.
Note: Tilt the plate so the embryos settle and remove the liquid from the top.
E. Replace the salicylic stock solutions with the appropriate fresh salicylic stock solution using a clean pipette each time.

F. Place the plate under a dissecting microscope and *record observations on the student data sheet. Note/describe any developmental markers and abnormalities*. **Repeat** for all salicylic acid concentrations.

G. Remove 1-2 embryos and place them on the depression slide with the cover slip. Observe the embryos using the compound microscope. *Record observations on student data sheet*. **Repeat** for all the salicylic acid concentrations.

H. Return the embryos to their wells in the plate.

I. Return the plate to the appropriate 28.5C incubator.

Day 3

A. **Repeat** day 2 work and observations. *Record all data*.

Day 4

A. **Repeat** day 2 work and observations. *Record all data.*

B. Place all embryos and fish in a waste container. The teacher will properly dispose of the organisms.

* upon the death of all embryos at 24 and 45 hours past fertilization new fish were exposed to more diluted concentrations of salicylic acid at these hpfs*

Above procedure and material list were taken from: SEPA Program- UW- Milwaukee

Data

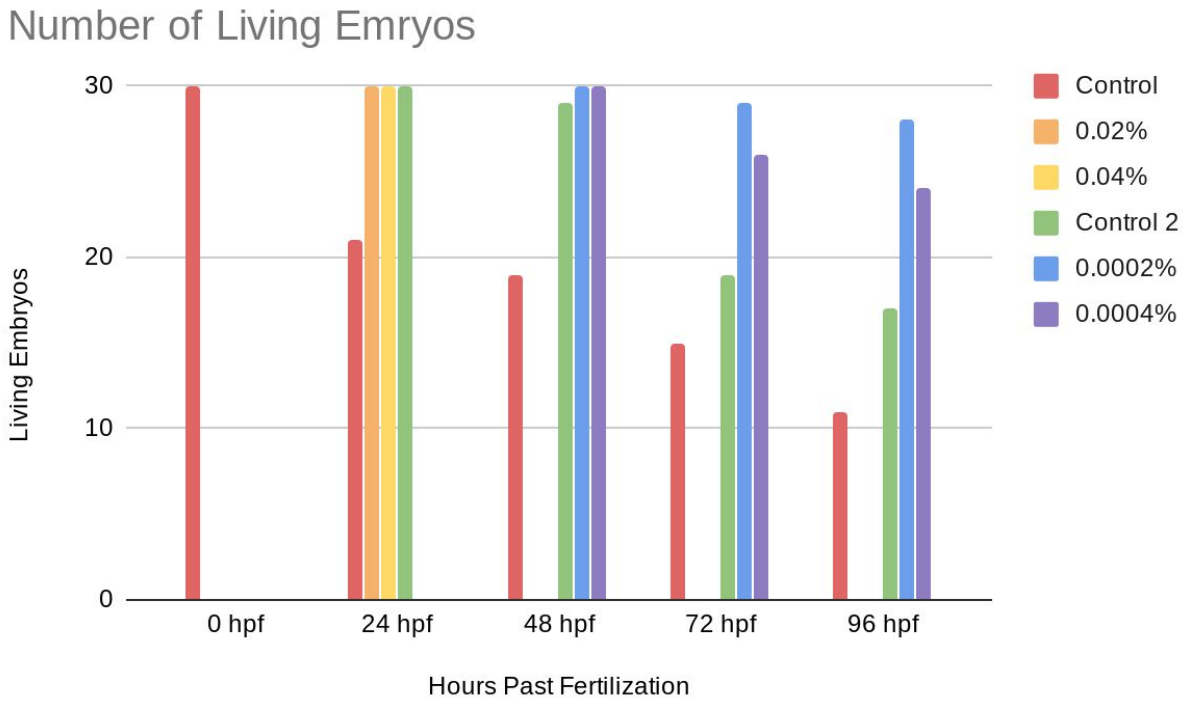
Data Table 1

Number of Living Embryos					
Concentration	0 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	30	21	19	15	11
2%	30	0	x	x	x
4%	30	0	x	x	x
6%	30	0	x	x	x
0.02%	x	30	0	x	x
0.04%	x	30	0	x	x

Control 2	x	30	29	19	17
0.0002%	x	x	30	29	28
0.0004%	x	x	30	26	24

Number of living embryos in various concentrations throughout the week

Figure 1



Number of living embryos in various concentrations throughout the week

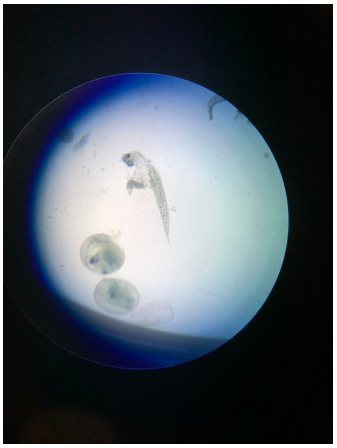
Figure 2

Zebrafish with a Spine, a defect found in the control well at 96 hpf.

Figure 3

A group of zebrafish at 72 hpf 0.0002% wells. Visibly curved healthy.

Figure 4



At 24hpf a group of dead zebrafish in the 4% well. All fish died in this well.

Figure 5

At 72 hpf well developed fish in the 0.0004% wells.

Data Analysis

A t-test was used with a p-value set at 0.05 to test statistical significance. An unpaired t-test is used to determine if two groups have different average values. This analysis allows predictions to be made from the data set.

Data Table 2

Significance of Living Embryos			
hpf	Comparison	P-Value	Significance
24	Control vs 2%	0.0292	statistically significant
	Control vs 4%	0.0292	statistically significant
	Control vs 6%	0.0292	statistically significant
Exposed at 48	Control vs Control 2	0.3632	not significant
	Control vs .02%	0.0168	statistically significant
	Control vs .04%	0.0168	statistically significant
Exposed at 72	Control vs .0002%	0.4522	not significant
	Control vs .0004%	0.3769	not significant
	Control vs Control 2	0.6331	not significant
96	Control vs .0002%	0.0795	not significant
	Control vs .0004%	0.2461	not significant
	Control vs Control 2	0.5322	not significant

The P-values and significance of survival rate data.

Much of the data was found to be significant, however the 0.002% and 0.004% concentration of salicylic acid at 72 and 96 hpf were calculated to be not significant.

Results

In this experiment zebrafish embryos were exposed to the independent variable, various levels of salicylic to see if there was a change in the dependent variable, the survival rate compared to that of zebrafish in the control. In the experiment 2%, 4%, 6%, 0.02%, 0.04%, 0.0002%, and 0.0004% concentration of salicylic acid were used. Over five days the embryos environment, whether it be the instant ocean or the solutions, remained unchanged. The goal of this experiment was to see the effects, if any of the salicylic acid on the survival rate of the zebrafish embryos. Much of the data was found to be significant.

Discussion

The data found partially supported the hypothesis, for there was an expected increase in fatalities, however the increase was drastic compared to what was anticipated. At 24 hours past

fertilization all fish died, and so the concentrations were diluted (0.02% and 0.04%). At 48 hours past fertilization once again all the fish died, and again diluted the solutions (0.0002% and 0.0004%), and this came as a surprise as in that Zinva's 2016 study zebrafish were found to be insensitive to the salicylic at these concentrations. At 72 hours past fertilization the fish lived, and with no visible deformities, making this data insignificant compared to the latter. And while the assumption can be made that the lower concentration is the cause for the fish surviving, their survival could also be attributed to the fact that they were exposed when they were more mature; this would justify the need for another study in which the fish be exposed to the lesser concentration at an earlier hpf or the more mature fish be exposed to the high concentrations.

With this study being as flawed as it was, through lack of time or cross contamination, there is much room for error and cause for question as to what in reality affected the fish in what way.

As the data did drastically jump from all the fish dying to an insignificant number dying another study should be conducted to test a greater quantity of salicylic acid concentrations to find the threshold at which the fish live, die, or have abnormalities.

There being data to prove that salycic acid increases the risk of fetal intracranial hemorrhaging would also warrant another study, if possible, to conclude the cause of death of the embryos.

There is very little current research that suggests that salicylic acid does have a negative impact on both zebrafish and humans, however the results of this study suggest that further research should be conducted.

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