The effects of ethanol on pigmentation development, dispersion, size, and shape in *Danio rerio* embryo heads

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Introduction:

*Danio rerio*, also known as the zebrafish, have been sought out in developmental biology studies of vertebrate species due to their ability to develop and mature quickly, as well as their adeptness in reproducing offspring within a short timeframe (Tyler, 2010). As an added bonus, zebrafish lay transparent eggs, which makes observing development, either normal or perturbed, relatively easy. *Danio rerio* are important to study for this very reason, since they can be used to closely monitor normal development versus stunted development to observe developmental change or malformations that occurred.

A sufficient amount of research has been done on zebrafish, especially in regards to teratogenic effects, such as alcohol exposure, on embryonic growth. Many of these experiments focus on visible and quantifiable measures, such as the effects of alcohol exposure on body length, distance between the eyes to test for cyclopia, as well as fin size and development. One area that is lacking in research is changes in pigmentation cells due to alcohol exposure. Of the few studies that have been done on the effects of alcohol on pigmentation cells, one conducted on the acute effects of ethanol on zebrafish embryos found, accidentally, that ethanol impacted the size and shape of melanocytes (Lockwood, Bjerke, Kobaya & Guo, 2004). These researchers found that the dilution of ethanol and the duration of the exposure were crucial factors in the appearance of the melanocytes, despite their internal presence within the dermis layer, not external presence, which must mean that the exposure of alcohol perturbed an externally and cascaded to impact the internal pigmentation cells (Lockwood et. al., 2004). Other research on the teratogenic effects of alcohol on zebrafish embryos found that the Wnt signaling pathway, which triggers β – catenin and is thought to be involved in the formation of neural crest cells, plays an important role in later formation of pigmentation cells (Dorsky, Raible, & Moon, 1999). A significant amount of time has passed since this pigmentation study run by Dorsky et al., but it was found that Wnt signals were necessary and sufficient for establishing pigmentation cell fates, which means it could very well play a role in the effects of early alcohol exposure on pigmentation growth.

In our study, we tested the effects of embryonic exposure to alcohol on zebrafish to test the hypothesis that pigmentation is impacted, either directly or indirectly, by the presence of ethanol, which creates a cascade event that can be seen in the form of a physical phenotype of less dispersed and more jagged-looking pigmentation cells on zebrafish embryo heads.
Materials and Methods:  

Zebrafish Model of Fetal Alcohol Syndrome

The lab procedure used for studying the teratogenic effects of embryonic alcohol exposure on pigmentation cells was designed in two parts, both by Steen et al. (2015). Three large fish dishes were obtained, which were used to hold three smaller petri dishes, each labeled appropriately with initials and alcohol concentrations 0%, 0.3% and 3% respectively. Each petri dish contained four zebrafish embryos in addition to 10 milliliters of fish water from their natural environment. This fish water was comprised of 60 mg of ocean water and 1 liter of sterile distilled water (Tyler, 2010). The levels of ethanol that were added to the fish water were in concordance to guidelines established by IACUC, the Institutional Care and Use Committee, to ensure that the minimal amount of ethanol concentrations would be used that are known to have an effect, rather than perturbing the zebrafish embryos more than necessary. The appropriate concentrations of ethanol were then added to each of the 0.3% and 3% labeled petri dishes, which was done by removing some of the fish water and then replacing it with the diluted ethanol. A dilution ratio for ethanol to water, 1:1, was utilized, meaning that 0.6% ethanol was diluted to achieve 0.3% ethanol and 6% ethanol was diluted to achieve 3% alcohol respectively. To be consistent and give all embryos the same environmental disturbance, fish water was removed via pipet and was then added back to the initial control groups. Since the ratio was 1:1, 5 milliliters were taken out of each petri dish, with the respective fish water or ethanol dilution being re-added once completed. Observations were made daily on the zebrafish embryos over the course of seven days, and the fish water containing the ethanol dilutions were replaced with clean fish water twenty-four hours after the addition of the ethanol.

Fixing and Staining Zebrafish Embryos

Once observations were complete over the course of the seven days, the remaining zebrafish embryos were anesthetized, fixed, and stained with Alizarin red and Alcian blue, along with 2-week post-fertilization control zebrafish, according to the procedure written by Steen et al. (2015), with the following modifications: the Alcian blue staining step lasted two hours and twenty-three minutes, no phosphate buffer saline, known as PBS within the procedure, was used, the Alizarin red staining step lasted one hour and thirteen minutes, the 20% glycerin step lasted seventeen hours, and lastly, the 60% glycerin step lasted eighteen hours. In total, there was one zebrafish embryo from the 0.3% condition, one from the experimental 0% condition, and six 2-week controls. The two zebrafish embryos that were in the experimental condition were compared against the six zebrafish embryos that were in the new control condition. Having the control condition was necessary to see if the exposure to alcohol for the twenty-four hour duration had a significant effect on the formation of pigmentation cells.
Observations of Fixed and Stained Zebrafish Embryos

By utilizing microscopes in the ICUC and accounting for Koehler Illumination, images of each zebrafish embryo head at a frontal, direct view were taken and used in data collection. The images of the zebrafish embryos were taken over the course of two separate days, using two separate microscopes. One of these microscopes utilized BTV to capture images while the other utilized SPOT to capture images, which resulted in the images of the zebrafish embryos exposed to 0.3% ethanol to look different from the other images of the zebrafish embryos. These images were used to analyze the dispersion, size and shapes of the pigmentation cells on the zebrafish embryo heads. In order to use these observations to create and analyze data, two concrete establishments were made by using a decoding sheet: differentiating between one or multiple pigmentation cells if they were in close proximity to one another relative to their sizes, and the different between jagged and circular pigmentation clusters. Once decoded, the relative sizes (small, medium, large) and the relative shapes (circular and jagged) were totaled and used to create two bar graphs in Microsoft Excel.

Results:

The analysis on the effects of the ethanol on zebrafish embryo pigmentation was conducted separately. While looking at each individual zebrafish embryo head, it was evident that a decoding sheet for various types of pigmentation cells would be necessary, as there was a lot of overlap between various pigmentation cells, in addition to a wide range of sizes which would need to be categorized definitively before beginning analysis. Figures 1A and 1B depict the basic decoding sheets for various types of pigmentation cells that were observed in this study, in relation to their overall shape. These decoding sheets were used when looking at each individual embryo, and were created by averaging commonly observed pigmentation clusters.

The images collected from the microscopy were analyzed in Adobe Photoshop to differentiate between the various: sizes (short, medium, long) and shapes (circular or jagged) of the pigmentation cells. The dispersion of the pigmentation clusters were analyzed based on the total number of clusters observed, relative to their shape and size. Individual melanocytes have been highlighted in red circles in Figure 2A-1 to 2H-1 on the right-hand side of the column. The original versions of the stained and fixed zebrafish head images can be seen in Figures 2A-2 to 2H-2 on the left-hand side of the column. Embryos 2A and 2B, which were in the experimental condition (n = 2), had the fewest amount of small pigmentation clusters (see Figure 3A), as well as the fewest amount of circular shaped pigmentation (see Figure 3B). The total amount of pigmentation on both the zebrafish embryos that were in the experimental treatment was also fewer than the average of the control embryos: 2C, 2D, 2E, 2F, 2G, and 2H (n = 6). Each zebrafish embryo from the control group, with the exception of 2F, which was albino and therefore had no pigmentation, had significantly more short and circular clusters of pigmentation.
Figure 1A. Image showing various, average examples of circular clusters of pigmentation cells. Used to decode samples in study.

Figure 1B. Image showing various, average examples of jagged clusters of pigmentation cells. Used to decode samples in study.

Figure 2A-1. Image showing zebrafish embryo in experimental group exposed to 0.3% ethanol at 40x magnification. In collaboration with Malik Zaza.

Figure 2A-2. Image showing zebrafish embryo in experimental group exposed to 0.3% ethanol at 40x magnification. Red circles represent pigmentation. In collaboration with Malik Zaza.

Figure 2B-1. Image showing zebrafish embryo in experimental group exposed to 0% ethanol at 4x magnification.

Figure 2B-2. Image showing zebrafish embryo in experimental group exposed to 0% ethanol at 4x magnification. Red circles represent pigmentation.
Figure 2C-1. Image showing zebrafish embryo in control group at 4x magnification.

Figure 2C-2. Image showing zebrafish embryo in control group at 4x magnification. Red circles represent pigmentation.

Figure 2D-1. Image showing zebrafish embryo in control group at 4x magnification.

Figure 2D-2. Image showing zebrafish embryo in control group at 4x magnification. Red circles represent pigmentation.

Figure 2E-1. Image showing zebrafish embryo in control group at 4x magnification.

Figure 2E-2. Image showing zebrafish embryo in control group at 4x magnification. Red circles represent pigmentation.
**Figure 2F-1.** Image showing albino zebrafish embryo in control group (in center) at 4x magnification.

**Figure 2F-2.** Image showing albino zebrafish embryo in control group (in center) at 4x magnification.

**Figure 2G-1.** Image showing zebrafish embryo in control group at 4x magnification.

**Figure 2G-2.** Image showing zebrafish embryo in control group at 4x magnification. Red circles represent pigmentation.

**Figure 2H-1.** Image showing zebrafish embryo in control group at 4x magnification.

**Figure 2H-2.** Image showing zebrafish embryo in control group at 4x magnification. Red circles represent pigmentation.
Figure 3A. Graph showing the size variation, small, medium or large respectively, in the sample size of zebrafish embryos. The total number of pigmentation clusters, used to determine dispersal of these clusters, can also be derived from this data.

Figure 3B. Graph showing the variation in shape, either jagged or circular respectively, in the sample size of zebrafish embryos. The total number of pigmentation clusters, used to determine dispersal of these clusters, can also be derived from this data.
Discussion:

Jagged shaped pigmentation were the least common of the two relative shapes, making circular clusters the most common. This observation was true in each treatment group, thereby making these relative sizes insignificant when compared against themselves. When circular shape and small size are analyzed together, it can be concluded that small, circular shaped pigmentation clusters are most commonly found in the zebrafish embryos that were not treated with any ethanol solution or received any environmental disturbance. The zebrafish embryos that were in the control group also had a higher amount of total pigmentation clusters than the zebrafish embryos that were in the experimental group.

The amount of jagged pigmentation clusters was not significant within any treatment group, which refutes the previously suggested hypothesis that pigmentation creates a cascade event seen in the form of a physical phenotype of less dispersed and more jagged-looking pigmentation cells on zebrafish embryo heads. On the other hand, it is important to note that there were significant differences between the two zebrafish embryo treatment groups, as there was a higher percentage of circular and small melanocytes in the control group than there were in the experimental group. This supports the first part of the proposed hypothesis, which states that pigmentation is impacted, either directly or indirectly, by the presence of ethanol.

To study the cascade effects of the ethanol on the formation of pigmentation, knock-out experiments to test for necessity and sufficiency should be conducted for the Wnt signaling pathway and sonic hedgehog. Wnt signaling pathways were found to be necessary and sufficient in the formation of pigmentation (Dorsky et al., 1999). Wnt signals help to establish the dorsal axis of the specimen, similar to sonic hedgehog, which is another mutagen (Omont & Kepes, 2005). It would be important to test the necessity and sufficiency of sonic hedgehog in creating the cascade event that impacts the formation of the pigmentation on zebrafish embryos.

Unfortunately, there were a few setbacks in this experiment. The sample size for this study was quite small, in addition to one of the control zebrafish embryos, 2F, being albino, making it an outlier and therefore unusable. In future experimentation, a larger sample size should be utilized; one that accounts for various levels of alcohol exposure and duration, as this study only investigated the effects of 0.3% ethanol over a twenty-four hour period. Another shortcoming of this study was the lack of zebrafish embryos that had been exposed to ethanol. Only one of the experimental zebrafish embryos had been exposed to alcohol, the other experimental zebrafish embryo had been disturbed an equal amount, but did not receive an ethanol concentration. This may say something about how environmental factors of the zebrafish can impact the size, dispersion, and shape of the pigmentation cells on their heads, but more research would need to be conducted to come to any conclusions.
References:


