Acute Effects of Alcohol on Development of Neural Crest Cell Development and Migration in Zebrafish (Danio rerio)

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Independent Research Project Report
Developmental Biology
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12/12/07

Introduction

Zebrafish (*Danio rerio*) are a beneficial model for embryonic development and especially Neural Crest Cell formation and the contributing factors that we will study. They have a high fecundity (females lay several hundred eggs at weekly intervals) and short generation time which allows for a quick turnover of several stages to an experiment (Ziello 2004). Further benefits of Zebrafish are their translucent embryos which enables easy observation with use of a dissecting microscope. Finally, Zebrafish also are easily maintained in a laboratory environment and develop very rapidly (Ziello 2004). The transparency of the embryo and the availability of mutants and fluorescent transgenic lines in the zebrafish allow a more sophisticated analysis of the routes the neural crest derived cells take during larval and adult development(Douarin 1999). For these reasons, Zebrafish has proven to be a beneficial subject for developmental studies and especially for neural crest cell migration and development.

Animal pigmentation patterns are one of the most notable observations made when initially observing a species. These patterns can serve a range of functions including camouflage, warnings (colors of poison arrow frogs), social aggregation and mate choice (Jeffery 2004). Because of their uniqueness and range of distinction, pigment patterns have become a primary source not just for observation but also for natural selection. The creation of pigmentation and its functions have been of particular interest not only to evolutionary biologists, but developmental biologists for their ease of observation and manipulation (Jeffery 2004). In more recent years, pigment patterns have become useful as a model of post-embryonic development in discovering why adult organisms look the way they do and further understanding their developmental morphology (Jeffery 2004). Pigmentation patterns have been especially useful for research covering multiple specialties within biology simultaneously including molecular, phenotypic development, and evolution from individuals, to general populations and eventually to entire species development (Jeffery 2004). For these reasons and others mentioned before, we have chosen to research pigmentation development and migration in the zebrafish, *Danio rerio*.

There are copious amounts of variations within species of pigment patterns which has been demonstrated with previous research of Zebrafish. The pigment cells that we associate with these patterns have an origin as neural crest cells that develop along the dorsal neural tube during neurulation and are actually one of the most dramatic examples of cell migration observed in developmental biology (Douarin 1999). Neural crest cells have additional destinations other than just pigmentation, which makes them of particular interest to understand the mechanisms involved in the pigmentation process, as well as the evolution and diversification of vertebrates. The pigmentation of adult zebrafish have an origin in three different types of cells; chromatophores: black melanophores (containing melanin); yellow or orange xanthophores (containing pteridines and carotenoids); and silvery iridophores (containing guanine-rich reflecting platelets) (Douarin 1999). The dark stripes that are most commonly associated with Zebrafish include melanophores and iridophores along with a small amount of xanthrophores. The light stripes, however, include xanthrophores and iridophores with only a minute amount of melanophores (Douarin 1999). Current molecular analysis of these cells are supporting the theory that there are many similarities in both pigmentation material and mechanisms for development (Douarin 1999).

Neural crest cells (NCCs) are pluripotent migratory cells that have proven to be crucial to the development of the peripheral nervous system, pigment cells, and craniofacial cartilage and bone (Douarin 1999). NCCs are specified within the dorsal ectoderm and undergo an epithelial to mesenchymal transition (EMT) in order to migrate to their final destinations (Douarin 1999). The neural crest is one of the most notable aspects of vertebrates and a crucial part of our every day function. It is these mechanisms that we hope to determine the factors of through exposure to alcohol at several concentrations. Our motivation for this is a result of the increasing number of Fetal Alcohol Syndrome (FAS) children who are affected by the increasing number of alcohol abuse in parents (CDC 2006). Alcohol is able to permeate the placenta and enter fetal circulatory system, thereby causing developmental abnormalities. Ethanol impairs placental blood flow to the fetus by constricting blood vessels: inducing hypoxia and fetal malnutrition (CDC 2006). Alcohol rapidly crosses the placenta and blood-brain barrier of the fetus and the damage produced depends on gestational period, dosage, and degree of chronic of abuse (CDC 2006). There are many proposed mechanisms of action for ethanol such as altered neural crest cell migration/increased neural crest cell death or general cell death by superoxide radial lysis of cells, mitochondrial cell dysfunction, may inhibit growth factors regulating cell proliferation and survival, effects on Glial cells, effects on development of neurotransmitter systems, effects on cell adhesion and altered developmental regulation of gene expression (NIAAA 2000). FAS is most characterized by abnormal facial features, growth deficiencies, and central nervous system (CNS) problems. More specifically, people who suffer from

FAS often have problems with learning, memory, attention span, communication, vision and hearing or any combination of these symptoms (NIAAA 2000). With the increasing rates of alcohol consumption and lack of education about the adverse effects, FAS has become one of the most common birth defects in the Western World (CDC 2006). The Center for Disease Control (CDC) reports shows a range of 0.2 to 1.5 children per 1,000 births in different regions of the Unites States (CDC 2006). With our prior knowledge and research on the effects of alcohol and fetal development, we expect to have similar results on the development and migration of Neural Crest Cells in the Zebrafish. Thus we have formulated the hypothesis that Neural Crest Cell (NCC) development in the Zebrafish will have a negative relation to the concentration of alcohol used resulting in decreased total count of NCC as well as inhibited migration.

Materials and Methods

This procedure was carried out by Reed Hollett and assisted by Ashley Furr during the month of November, 2007.

A collection of both Male and Female Zebrafish, *Danio rerio* were placed in a small tank with marbles with natural pond water. We placed marbles at the bottom of the tank in order to prevent the embryos when they are fertilized from being eaten by the adult Zebrafish. The eggs were collected by using a manual siphon and placed in a large dish with several inches of the water from the tank. In order to maximize the number of embryos collected the siphon was kept close to the bottom of the tank and slowly stirred the marbles to achieve access to the embryos. This process was performed once on day 1, and again on day 2 to achieve embryos at two different stages of development. Embryos were then analyzed using compound microscopes on 4x magnification to determine whether they were at 24hr or 48hr development. Sample photographs were taken of each group to record the original development and provide a reference for further analysis. We used the imaging program Btv Pro through dissecting microscopes on magnification 5. Enough eggs were collected to divide each group further into three groups with 10 eggs each. The total number of embryos used was 60 with 30 in both 24hr development and 48hr.

Each group of 30 embryos was then divided into three 110ml petri dishes with a total of volume of 20ml. We then created a 2% stock solution of Ethanol (EtOH) to dilute into final solution for desired concentrations. To make the solution we added 0.2mls of 100% EtOH to 10mls of pond water resulting in a 2% stock solution. In the 24hr group, one petri dish was left unaltered to create a control group. We added 0.200mls of 2% EtOH to create our 0.02% EtOH solution and 0.02mls of EtOH to the third group to create the 0.002% EtOH group. Refer to the examples below for

further information on how to create EtOH solutions.

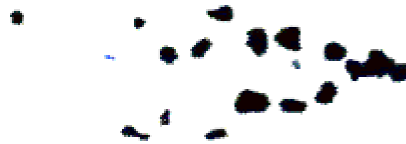
After the solutions were divided into the petri dishes, 10 Zebrafish embryos were taken from the 24hr development group and placed into each petri dish. It was important to do this after the solutions were created as to not expose any embryos to improper solutions. We repeated these steps for the 48hr embryos. The Petri dishes were then capped to slow the evaporation process and labeled Control24, Low24, High24, Control48, Low48, and High48 respective to their concentrations.

After 6 days of exposure the embryos were observed generally for patterns of development. We then selected two embryos from each petri dish to be placed in isolation and observed for individual developmental patterns. Each embryo was transplanted using a small pipette and submerged in ample amounts of their original solution and placed in their own petri dish. Digital images were then taken using a dissecting microscope and Btv Pro imaging program. The embryos were transferred using a small pipette and no cover slip was necessary. It is important to make sure there is amble amount of water on the slide to keep the fish in proper position to observe the dorsal side. Each embryo was observed on magnification 5. When capturing the images, it is important to make sure the entire dorsal side of the embryo is exposed for further analysis of the Neural Crest cells.

After initial observation, the photos were analyzed using the program Photoshop. Using the magic wand tool on the toolbar, I selected one of the black spots on the backside recognized as the neural crest cells through the research connecting pigmentation to NCC development. Refer to the pictures in the results section for examples of cells. To select additional cells, hold down the ‘shift’ key and click on another section. Only cells contained in the head were selected for analysis (head is defined as the body structure anterior to the stomach bulge). After all neural crest cells were selected, click on ‘image’ at the top of the screen and select ‘histogram’. This provided a total count of the pixels within the selected cells and this will represent the concentration of neural crest cells. If selection for each spot does not cover desired region, continue to hold ‘shift’ and click the spot additional times until the entire desired section is highlighted. This process was repeated for the following two days. The average from each of the two selected embryos were then averaged and graphed to allow further analysis.

Example of isolated NCC after selection

Section of highlighted using Photoshop same fish anterior to dotted line in previous picture.



Results

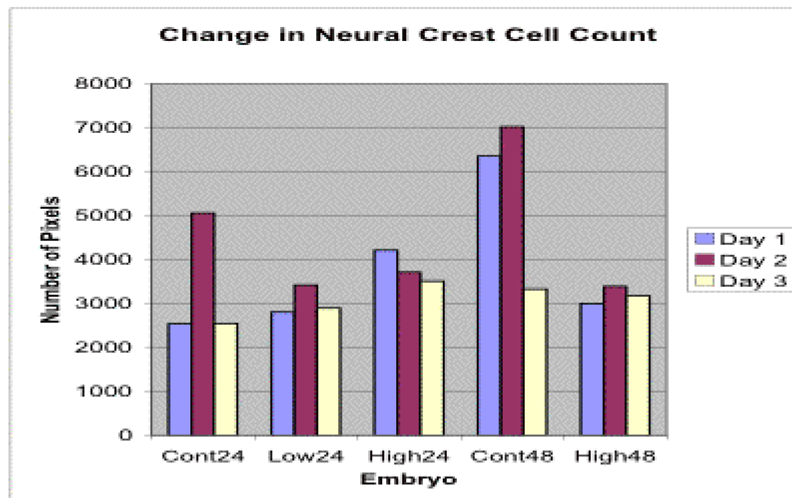


Figure 1 – This graph depicts the change in Neural Crest Cell count over the three days of measurement as represented by the number of pixels they occupied in the digital images.

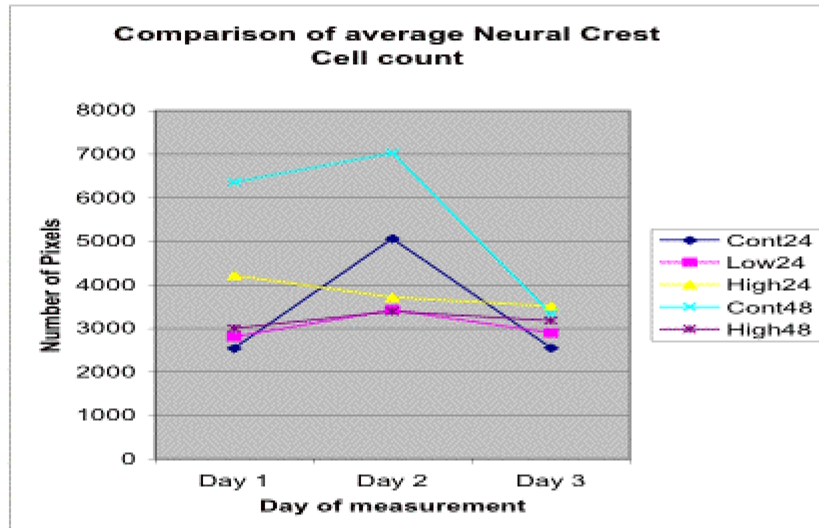


Figure 2 – This graph depicts a comparison between concentration groups during each day of measurement.

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Figure 3 – This picture represents an example of Zebrafish development at roughly 72hrs without exposure to EtOH.

Figure 4 – This picture represents a collection of Zebrafish embryos without EtOH exposure at roughly 72hrs of development.

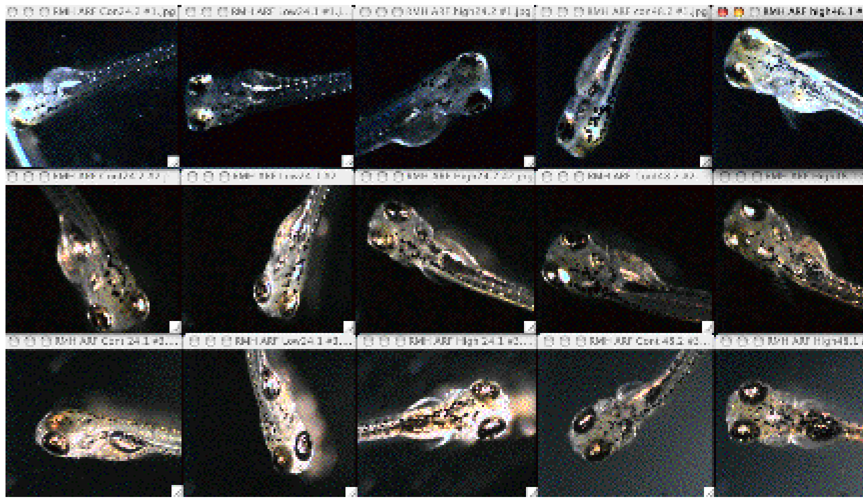

Figure 5 – This collage depicts samples from each concentration group on each day. Of the two fish used for experimentation in each concentration group, the digital image that best represented the days measurements was selected for this collage. The rows represent the days of measurement chronologically, and the columns are Cont24, Low24, High24, Cont48, and High48.

As seen in figure 1, which depicts the average NCC count over the three days of measurement, there were no distinct patterns in the development and migration of the cells. Figure 4 also shows individual patterns of growth over the three days and each set of embryos except the High24 group showed a peak cell count on day 2. Both control groups also represented the highest total cell counts out of all the embryos, both of which occurred on day 2. The 48hr development group showed the greatest separation in NCC count from the control to the high concentration group.

Figure 2 represents the change in NCC count over the three days of measurement as well as a comparison of each concentration group. The Cont48 group yielded the highest overall cell count by more than 2000 pixels, and was the highest overall throughout the three days. The low24 group was most consistently the lowest cell count with hardly any change through each day of measurement and a maximum cell count of less than 3500. The Cont24 group had the most significant jump in cell count during day 2 with a change of more than 2500 pixels and a return to the original count on day 3.

Figure 1 shows that 3 out of 5 groups stayed within a range of 500 pixels (unit of measurement for total NCC count) throughout the three days of measurement. The Cont24 group showed a significant peak on day 2 and the Cont48 group had the most significant alteration moving into day 3. As depicted in Figure 2, all the concentration groups had a maximum spread of almost 4000 pixels, but were all within 1000 pixels for the final day of measurement. Finally, as seen in Figure 4, the patterns of NCC are more compact than those exposed to EtOH which appear to be more spread out.

Conclusion

Overall the experiment was successful in the sense that quantifiable data was obtained and patterned variation within experimental groups occurred. Although the hypothesis was not able to be completely supported, several beneficial conclusions were able to be drawn from the data. The most general conclusion was that EtOH did appear to have effect on the NCC development as the Control groups yielded significantly higher numbers in pixel count over all of the EtOH concentration groups. This difference was most notable on day 2 of measurement which was exactly 1 week after initial exposure to EtOH. It is important to note here that measurements were only taken at the most anterior region of the embryo to include the head and exclude the stomach and tail regions. Observations were also only taken on the dorsal side of the fish, both of which are important because it is not the total number of NCC that is represented but only within this region so intense migration towards other regions may account for the fluctuations in numbers. Interestingly, however, the most extreme fluctuations only occurred in the control groups, whilst the EtOH groups maintained a relatively stables count throughout the three days of measurement. This may be used in further studies to determine specific days to track migration of NCC and possibly how EtOH may inhibit this process.

As mentioned in the results section and depicted in Figure 4, three out of the five groups maintained a relatively stable cell count except for the Controls which each showed a dramatic jump during the final two days. This leads towards conclusions about the effects of EtOH and developing new NCC as well as inhibiting migration. As seen in Picture 2, NCCs are already visible at 72hrs of development. Neural development has already begun at the point of 48hr development and thus remains unaltered at this point for the experiment but may be altered in further testing.

The difference between the two control groups is most significant in days 1 and 3. Since all other groups were relatively the same in comparison between 24 and 48 hours of development, the control groups are the only representation of altered development dependent on critical periods of exposure for NCC development. An earlier exposure to EtOH seems to have a greater effect on inhibiting the NCC development than at 48hrs. It would be very beneficial to design an extension of this experiment to test exposure at specific hours after fertilization and further along development after the 24 and 48 hour groups used in this one. The concentrations at these intervals seemed to be a non-factor overall, though the pattern of development was actually opposite than proposed in the hypothesis. There was an increase in average NCC count from the Low24 to High24 group. Unfortunately there were no surviving embryos from the Low48hr group so it is impossible to determine any relationship there.

Although this experiment proved to be very successful, there are several things that would be important to pay more attention to and alter if additional experiments were designed based on this one. The most important change to be made would be to refresh the EtOH solution each day or at least a couple times throughout the experiment to maintain a

more constant exposure as the EtOH will evaporate faster than the water. As a result, this experiment only mimicked a single alcohol exposure rather than chronic abuse as seen in most FAS patients.

References

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