The Effect of ethanol concentration on beta-cell development in zebrafish

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Summary

Type 1 diabetes is a disorder in which pancreatic β-cells do not provide sufficient insulin to moderate blood sugar levels. Fetal alcohol syndrome is a condition caused by alcohol consumption during fetal development that results in a host of developmental defects. The purpose of this study is to assess the effect of ethanol (EtOH) on β-cell development in developing embryos. Zebrafish embryos were exposed to concentrations of EtOH diluted in embryo medium. Six experimental groups were created by exposing groups of 50 embryos to 0.10%, 0.25%, 0.75%, 1.00%, and 1.50% EtOH, with 0% EtOH serving as a control. The embryos were then rated based on the amount of β-cell degradation in the embryonic pancreases on a scale of zero to four, ranging from no β-cell damage to extreme β-cell damage. We show that higher concentrations of EtOH caused increased damage to β-cells, with average grades of the embryonic β-cells increasing with EtOH concentration: average grades for 0%, 0.10%, 0.25 %, 0.50%, 0.75 %, 1.0 %, and 1.50% EtOH exposures were 0.71, 0.61, 0.55, 1.0, 0.82, 2.1, and 4.0 respectively. Therefore, exposure to increasing amounts of EtOH during pancreatic development may increase the severity of primary β-cell degradation. This study suggests that if a woman were to drink alcohol during the first trimester of her pregnancy, her child would be at a higher risk of developing type 1 diabetes.

Introduction

Fetal alcohol syndrome (FAS) is a condition that occurs in children whose mothers consume alcohol while pregnant. This syndrome causes many birth defects including but not limited to irregular growth (pre- and post-birth), irregular muscle control, mental retardation, behavioral issues, and heart defects (1). Miscarriages and stillborns, premature delivery, and sudden infant death syndrome (SIDS) are also very common amongst cases involving FAS (2). Also, there is a known correlation between FAS and insulin deficiencies, or type 1 diabetes, in newborn rats (3). FAS occurs mainly when the mother drinks a significant amount of alcohol during the main developmental stages of pregnancy.

This study investigates the correlation between FAS and type 1 diabetes, a β-cell disorder. The endocrine system controls most body functions through a series of regulated hormone pathways throughout the body. One important function of the endocrine system is to regulate blood sugar levels through signaling pathways in the pancreas. The endocrine half of the pancreas is separated into four major types of structures: α-cells, β-cells, δ-cells, and pancreatic peptides. α-cells are responsible for increasing blood sugar levels by releasing glycogen, a structure that stores glucose, into the bloodstream, while β-cells

decrease blood sugar by releasing a hormone called insulin, which aids in the movement of glucose into cells from the bloodstream. Blood sugar levels are regulated by δ-cells, which control the activity of both the α-cells and β-cells. Type 1 diabetes, a disorder that results in an inability to regulate blood sugar levels, can be caused by two factors: either an inherent resistance to insulin in the body or a primary β-cell degradation. Although the cause of this degradation is currently unknown, researchers believe that type 1 diabetes is an autoimmune disorder, caused by a virus implanting itself into solely the β-cells, resulting in the attack of β-cells by the immune system (4).

The zebrafish (Danio rerio) is a heterotrophic fish species currently emerging as a model for toxicology research for multiple reasons (5). Zebrafish have many readily available mutant phenotypes that resemble an assortment of human diseases. Because their eggs are transparent, zebrafish embryos can be observed throughout their development and a wide array of developmental conditions can easily be observed. It is also easy to observe these disorders in the fish as transparency can be induced in them through exposure to the chemical phenylthiourea, making the observation of organ development easy in comparison to mice. The species also has a short generation time and reproduces in high quantities, making it easy to obtain large amounts of data (6). However, what makes the zebrafish most suitable as a model for mammalian studies is the fact that zebrafish organ systems grow in a fashion analogous to mammalian organ development, making zebrafish a better model for mammalian development than fruit flies. The main drawback of the zebrafish as a model is that, because birth is extra-placental, one cannot monitor the interactions between mother and child that are present in most mammalian species (7).

Another useful tool in genetic research, especially in monitoring internal body systems of animals, is the use of fluorescent proteins (fluorophores) to observe developmental progress of specific cells. The gene needed to express these fluorescent proteins can be easily inserted downstream of the promoter sequences for most cells and, after a few generations of breeding, one can have a population of animals expressing these proteins in specified cells. The proteins, when exposed to certain wavelengths of light, become excited and can therefore be seen under a fluorescent light microscope (8). Of the known fluorophores, two have become quite popular amongst the scientific community for observing cell growth and tracking the movement of fluids throughout the body: the mCherry protein and the green fluorescent protein (GFP) (9,10). Both of these proteins are stable when excited, not exhibiting the phototoxicity (toxic when excited by light) of other fluorophores. They can also both easily be seen soon after transcription when exposed to the proper wavelengths: mCherry is excited by wavelengths of 587-610 nm, producing an orange light, while GFP are excited by wavelengths of 475-510 nm, producing a green light. This experiment uses mCherry to track the development of β-cells in the developing pancreas of zebrafish embryos after exposure to ethanol (EtOH, 11).

The purpose of this experiment is to observe if there is any correlation between FAS and type 1 diabetes. In an adult, alcohol is metabolized mainly in the liver, pancreas, stomach, and brain (12). EtOH metabolism begins when the protein alcohol dehydrogenase IB catalyzes the reaction in which EtOH becomes a toxic compound called acetaldehyde. The acetaldehyde is then turned into acetic acid by ascorbic acid and the protein aldehyde dehydrogenase, which is expressed by cells in the liver, pancreas, stomach, and brain. However, developing embryos do not have fully functioning organs to efficiently metabolize this alcohol. The introduction of alcohol to the developing embryos may disrupt the catabolic pathway used in EtOH metabolism. By exposing the developing β-cells to increasing amounts of alcohol, it has been thought that amounts of acetaldehyde would increase to concentrations that could not be quenched by ascorbic acid levels and aldehyde dehydrogenase in the developing pancreas, since embryos metabolize ethanol at a lower rate than adults (13). We hypothesized that with increasing embryonic exposure to EtOH, the fish would experience increasing β-cell deficiencies, which could be monitored by observing mCherry protein production in the β-cells, as the promoters for mCherry expression had been inserted into the β-cell genes. The expression of mCherry in embryonic zebrafish β-cells was done by inserting the mCherry promoter between the ins gene and the ins promoter in parent fish using transposon-mediated DNA integration. Therefore, the expression of insulin is linked with the expression of mCherry, so without production of insulin, there will be no mCherry expression (14). These β-cell deficiencies would translate to a higher risk of diabetes in children who were victims of fetal alcohol syndrome.

Results

An initial experiment was performed to determine the optimal range of EtOH concentrations to use for the following experiment, which tested the effect of EtOH on zebrafish embryo β-cell development. The initial experiment used EtOH concentrations ranging from 0.00% to 2.00% EtOH in embryo medium. Fifty embryos were incubated in each EtOH concentration: 0.00%, 1.00%, 1.50% and 2.00% EtOH in embryo medium. The embryos were incubated in the respective EtOH solutions for two days and then removed and β-cell development was assessed. All embryos died after exposure to these EtOH concentrations, prior to observing β-cell development. The embryos exposed to 0% EtOH also died, indicating problems with the embryo medium during dilution of the stock solution. In addition, the embryos that were observed, though not living, were physically too disrupted to be use in later experiments, suggesting concentrations of EtOH ranged too high and the exposure time was too long. As a result of this experiment, all solutions were remade and the range of EtOH concentrations was changed to: 0%, 0.10%, 0.25%, 0.50%, 0.75%, 1.00%, and 1.50%. Time of exposure to EtOH was also changed to eight hours starting at 12 hpf to coincide with development of the embryonic pancreas.

As was expected, 82.4% of the control group was deemed grade 0 (e.g. Figure 1), meaning that their β-cells were healthy and could be observed as one tight group of cells, while 17.6% had lost its fluorescence due to photo-bleaching and was deemed grade 4 (Figure 5). In the 0.10% EtOH exposure,

the percentage of grade 0 fish was 82.1%, with 4% grade 1 (Figure 2). In the 0.25% EtOH exposure, grade 0 decreased to 71% of the entire population, while grade 1 rose to 16.1% and grade 2 (Figure 3) was 6% of the population. In the 0.50% EtOH exposure, percentages of grade 0 fell to 52.1% with the percentage of grades 1 and 2 rising to 25% and 8.3% respectively. In the 0.75% EtOH exposure, grade 0 rose to 63.6% and grade rose 1 to 15.2%. In the 1.00% EtOH exposure, percentage of grade 0 fish fell to 7.4% while grades 1, 2, and 3 (Figure 4), rose to 31.5%, 25.9% and 13% respectively. In the 1.50% EtOH exposure there was no fluorescence observed suggesting 100% grade 4 fish. If the trend of ~20% grade 4 due to fluorescence expression error persists, then the conclusion can be drawn that ~80% of the 1.5 % fish experienced complete β-cell death (Figure 6). Average grades were calculated to show a trend. The average grade of the fish remained around .75 until rising in the 1.00% EtOH and 1.50% EtOH exposures (Figure 7).

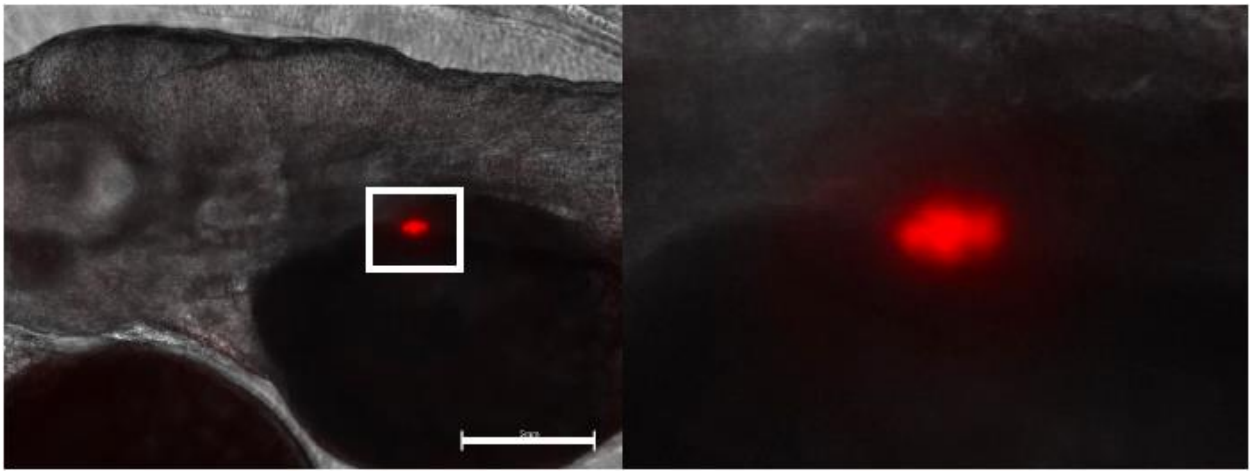

Figure 1. Grade 0: β-cell cluster prominent and organized, similar to a fist. Expected to be performing at peek efficiency for insulin production. Scale bar 5 mm.

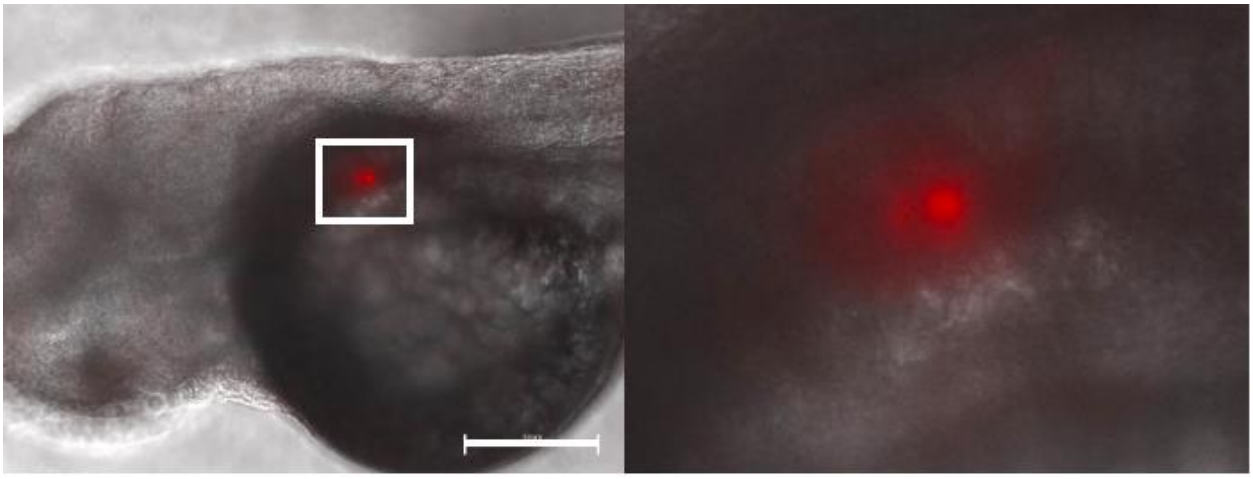

Figure 2. Grade 1: β-cell starting to detach from the main cluster. Expected to perform slightly less efficient than the Grade 0 formation. Scale bar 5 mm.

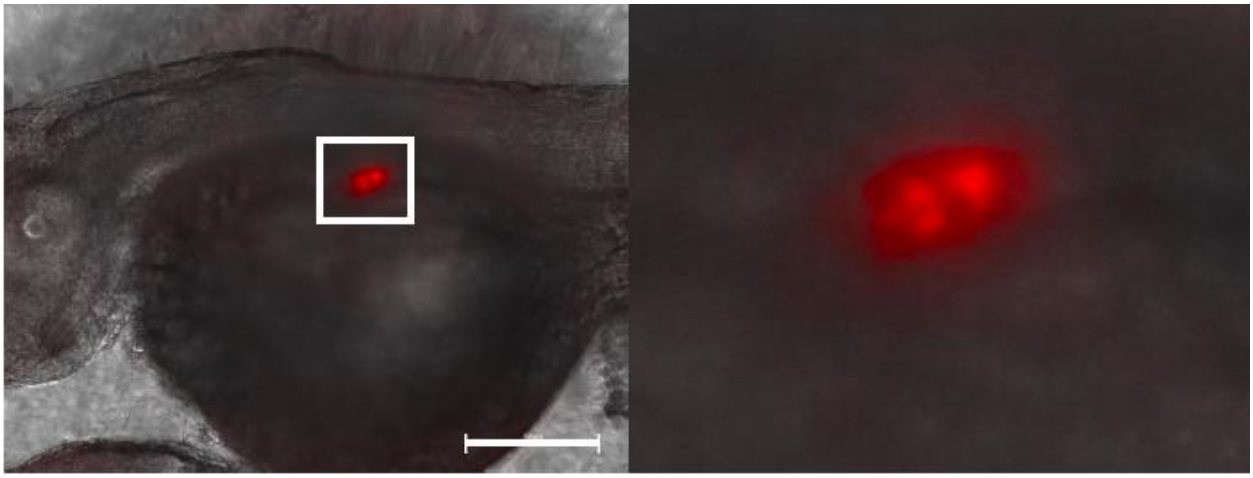

Figure 3. Grade 2: Two distinct β-cell clusters separate from each other. Area of dead cells dividing formations. Expected to perform less efficiently compared to Grade 0.

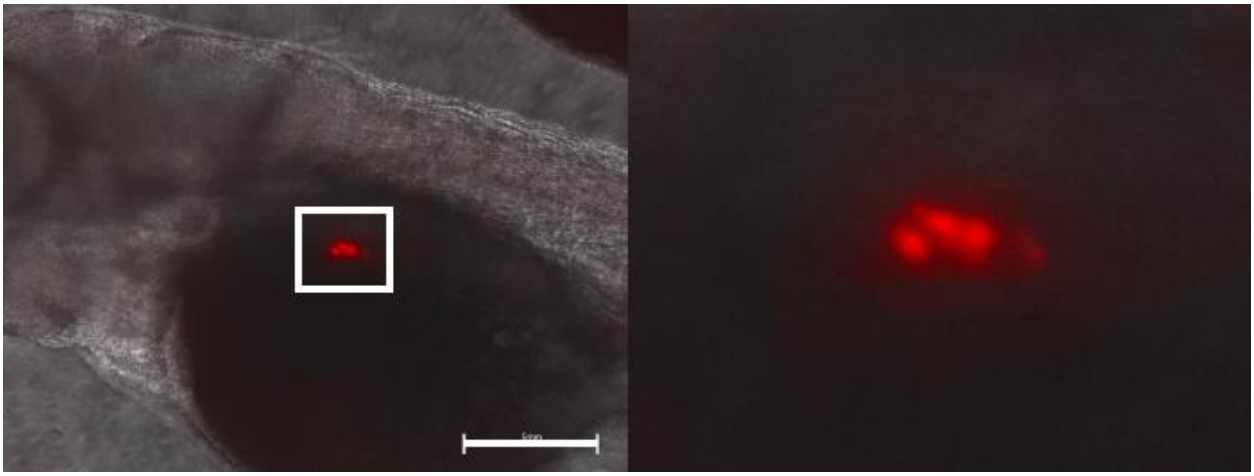

Figure 4. Grade 3: Linear formation of β-cell cluster. Significant dark area between each active cell. Expected to perform significantly less efficiently compared to Grade 0.

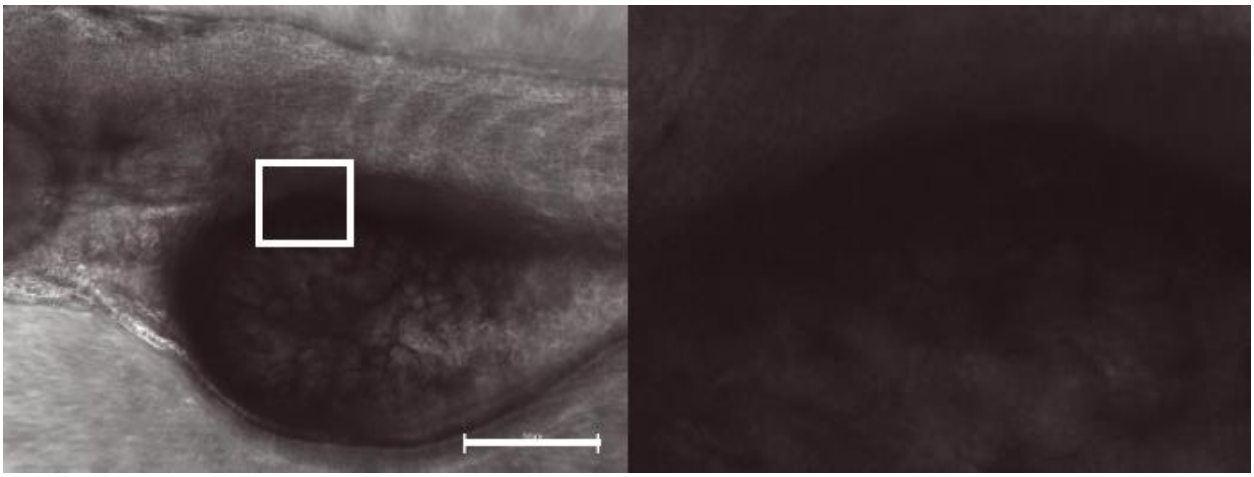

Figure 5. Grade 4: No fluorescence observed in the pancreatic region. B-cells are not producing the mCherry fluorescence but could still be producing insulin. Or, β-cells are dead and inactive in producing both fluorescence and insulin.

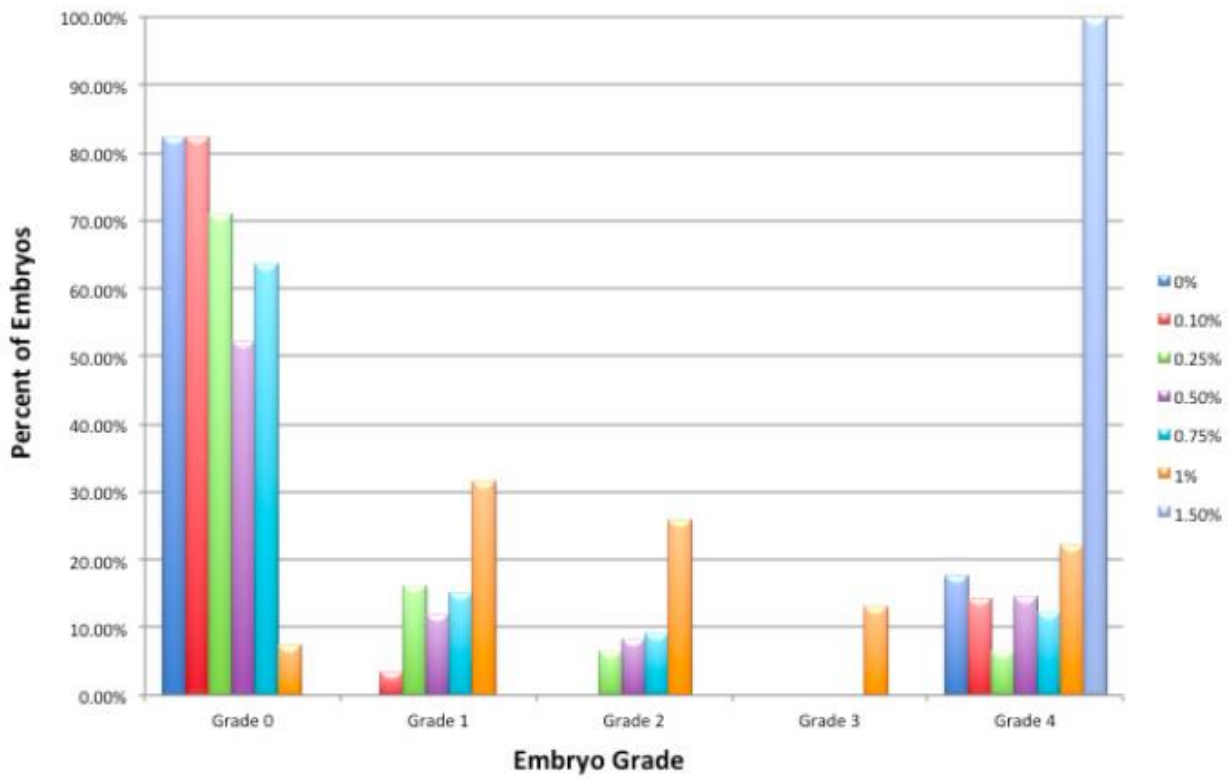

Figure 6: Effect of increasing concentrations of EtOH on percentage of embryos in each Grade. Percentage of Grade 0 embryos decreased, while percentage of Grades 1, 2, and 3 embryos increased with increasing ethanol concentrations.

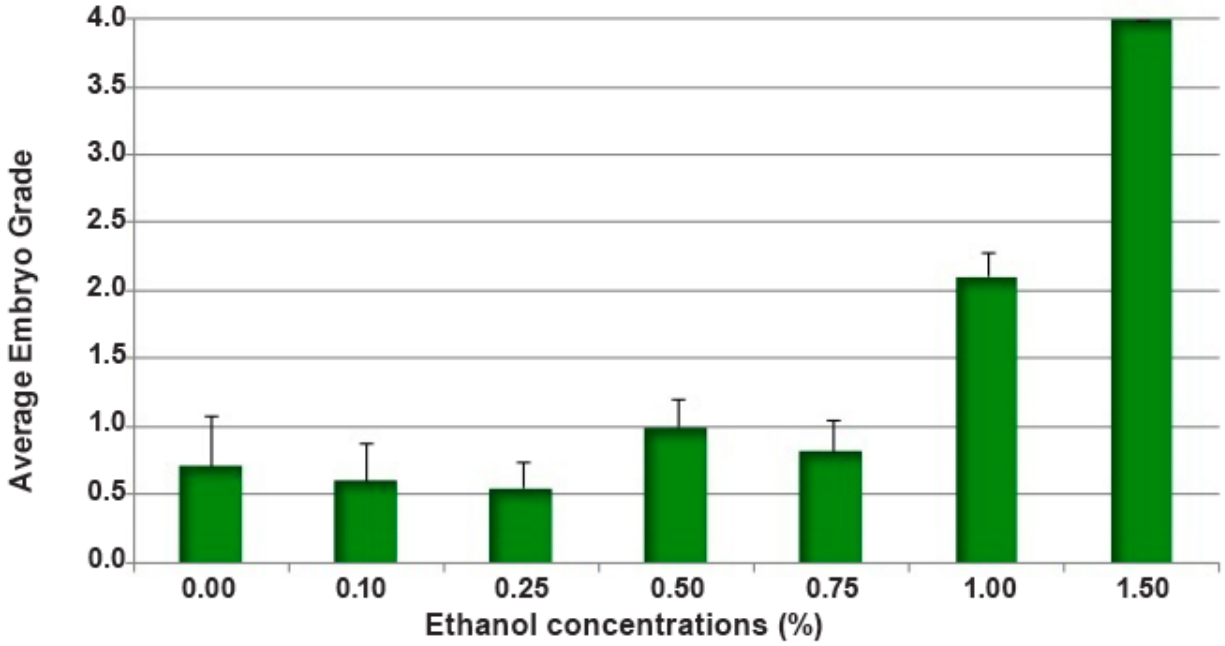

Figure 7: Effect of EtOH Concentration on β-cell Development. Error bars represent standard error, or standard deviation from the mean. P-values for 0%, .1%, .25%, .5%, .75%, 1%, and 1.5% ethanol are 1, 0.834, 0.836, 0.502, 0.804, 0.003, and 2.01×10&#x207B&#x2077 respectively.

Discussion

This experiment tested the effect of varying concentrations of EtOH on the development of primary β-cells in embryonic zebrafish. While there were some discrepancies with the control group due to some fish failing to express mCherry, the vast majority of the controls displayed the perfect grade 0 β-cells that were expected. Also, after calculating the percentage of β-cell deaths, it was found that the β-cells followed the expected pattern; the β-cell death rates increased at higher concentrations of EtOH, meaning the embryos exposed to higher concentrations of EtOH were at higher risk of diabetes. The same effect should translate over to humans and other mammals with analogous genomes to the zebrafish, meaning that if a woman were to ingest high concentrations of EtOH during the first trimester of her pregnancy, then her child would be at a higher risk of developing childhood diabetes, though the risk would depend on the amount of EtOH the woman ingested.

The final experiment, being based on qualitative observation, could have been subject to some error. The main problem that could have occurred is the simple mislabeling of some embryos based on an unclear image or the reflection of a cluster of β-cells on the final image of the embryos. The experiment was also based on human bias, as many fish were some intermediate between the extremes by which the grades were categorized. However, these intermediates usually leaned towards one of the grades over the others and were therefore labeled as the grade they most closely resembled. Also, during the experiment, all embryos, even those not being imaged, were exposed to varying amounts of light while

other fish were imaged. This exposure could damage the mCherry proteins and result in a lack of fluorescence, leading to these fish being categorized as “Grade 4” fish, since no β-cell activity could be recorded.

There are a few questions that can be drawn from this experiment, some perhaps even leading to future experimentation. When looking at the gathered data, one can see the slow drop in grade 0 fish and the increase in grades 1, 2, 3, and 4 fish with exposure to increasing concentrations of EtOH; however, this trend accelerates when one looks at the fish exposed to 1.00% and 1.50%, as all of the subjects in the 1.50% exposure were deemed grade 4 fish. Therefore, a future experiment could be done to see the β-cell development of the zebrafish exposed to concentrations of EtOH between 1.00% and 1.50%. Also, as discussed earlier, the zebrafish proves to be an excellent model for studying human genetics and morphology, a drawback being that zebrafish have extra-placental births. Therefore, the zebrafish eggs were submerged completely in their EtOH solutions. This however is not the case with placental birth, and while other experiments have been done showing the concentrations of EtOH that create analogous conditions to human FAS on zebrafish, none have been done on β-cell development specifically. This lack of knowledge means that the concentrations of EtOH used in this experiment may not be analogous to the conditions of human birth in terms of pancreatic development. One future experiment could involve the use of mammalian subjects actually ingesting EtOH as a human does, so one could measure the effects of the EtOH on the β-cells of placental born offspring.

Methods

To facilitate raising newly fertilized embryos, embryo medium was created. This was done by making a 1 L 60x concentrated stock solution by mixing 17.20 g $NaCl_2$, 0.76 g KCl, 2.90 g $CaCl_2 \bullet 2H_2O$ and 4.90 g $MgSO_4 \bullet 7H_2O$ [Sigma Aldrich]. The solution was then diluted to 1x concentration by adding 17 mL of embryo medium to 983 mL of distilled water. Then the 0%, 0.10%, 0.25 %, 0.50%, 0.75 %, 1.00 %, and 1.50% EtOH solutions were created.

Zebrafish, provided by the University of Massachusetts Medical School, were genetically modified to express the mCherry fluorescent protein in live, active β-cells. Parent fish were crossed by adding three adult female and six adult male zebrafish in a specialized breeding tank. The tank consisted of a bin with a grated bottom inside a bin with a solid bottom filled with embryo medium, as defined above. Fish were put on a 14 hour light and 10 hour dark cycle during mating. The resulting eggs were collected at the beginning of the next light cycle and were probed for healthy embryos. Eggs were placed in Petri dishes and observed using a Leica stereo light microscope. Embryos that were determined to be healthy were pipetted into six well plates, 25 embryos per well. Those that were determined to be unhealthy were discarded. Health was evaluated based on amount of sheen on the developing embryonic sack. The embryonic sacks that were shinier or clouded were unhealthy. Having filled eight wells, the

embryos, now at 12 hours post fertilization (hpf), were then submerged in 3 ml of their determined solutions. Two wells (50 embryos) were used for each solution. After eight hours, the embryos were taken out of their solutions and were submerged in 3 ml of 1x embryo medium and 0.2 mM phenyltheourea to prevent pigmentation.

At 3 days post fertilization, the embryos were fixed in 4% paraformaldehyde solution in phosphate-buffered saline and left overnight. Embryos were washed twice by transferring them to Eppendorf tubes containing fresh embryo medium until being placed on slides for observation. The embryos were then observed under a Nikon TI Eclipse Inverted light microscope, with a Sola fluorescent LED light source. This light source excited the mCherry fluorophore, which allowed the active cells to be viewed under the microscope. To establish that the mCherry expression was not effected by paraformaldehyde fixation, single embryos were observed after EtOH treatment prior to fixation. There was expression of mCherry indicating no negative effects due to the paraformaldehyde.

Grades were determined to quantify the severity of EtOH damage on the β-cells (See Figures 1-5). While grading was not done blind, each embryo was graded multiple times by various investigators independent from one another. Based on the organization of mCherry expressed in the pancreas, one can qualitatively determine β-cell functionality. Grade 0 was defined as a tight, centralized cluster of actively producing β-cells (Figure 1). Grade 1 was defined as a tight group of β-cells with a small group of cells detaching from the main group (Figure 2). Grade 2 was defined as two distinct groups of cells or two groups of cells with a third group beginning to form (Figure 3). Grade 3 was defined as either a complete disruption of organization in the cells, or three small clusters of cells (Figure 4). Finally, grade 4 was defined as an absence of fluorescence observed, whether that was due to the mCherry promoter wearing out under environmental factors, or complete β-cell death (Figure 5).

An unpaired, two-tailed t-test was performed using Microsoft Excel (Excel for Mac 2011), using each embryo as a single data point. Each set of data was compared back to the control group, with unequal variances assumed. Statistical analysis determined if the differences between the mean fish grades was significant or just due to chance. It also quantified the variation, or standard deviation, within the groups. The t-test showed statistically significant differences between the means of the 1.00% EtOH and 1.5% EtOH groups based on deviation from the control group.

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References

1. “Fetal Alcohol Syndrome.” A.D.A.M. Medical Encyclopedia. Ed. David Zieve and David R. Eltz. N.p.: n.p., 2012. N. pag. Print. <http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001909/>

2. Cohen, Robert M., and Bette K. Idemoto. “Diabetes and the Body: Pancreatic Function.” Net Wellness. N.p., 7 Dec. 2012. Web. 19 Dec. 2012. <http://netwellness.org/healthtopics/diabetes/pancreasdiabetes.cfm>.

3. Sadri, Parissa, and Dallas J. Legare. “Fetal Ethanol Exposure Causes Hepatic Insulin Sensitizing Substance-dependent Insulin Resistance.” Canadian Journal of Diabetes: 239-47. Print. <http://www.diabetes.ca/files/EthanolExposureLauttSept03.pdf>

4. “The Pancreas and Its Functions.” Columbia University College of Physicians and Surgeons. N.p.: n.p., n.d. The Pancreas Center. Web. <http://pancreasmd.org/education_home.html>.

5. Westerfield, Monte, P.I., and Doug Howe. “ZFIN: The Zebrafish Model Organism Database.” ZFIN. N.p., 1994. Web. <http://zfin.org/>.

6. Badman, David, et al. “Zebrafish FAQs.” Zebrafish FAQs. N.p., n.d. Web. 19 Dec. 2012. <http://www.neuro.uoregon.edu/k12/FAQs.html>.

7. Briggs, Josaphine P. “The Zebrafish: A New Model Organism For Integrative Physiology.” American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 282 (2002): R3-R9. Print.

8. “Excitation and Emission of Green Fluorescent Proteins.” biotek.com. N.p., 20 Feb. 2001. Web. 23 Feb. 2013. <http://www.biotek.com/resources/articles/green-fluorescent-proteins.html>.

9. “MCherry Fluorescent Protein.” ClonTech. N.p., n.d. Web. <http://www.clontech.com/US/Products/Fluorescent_Proteins_and_Reporters/Fluorescent_Proteins_by_Name/mCherry_Fluorescent_Protein>.

10. Shaner, Nathan C., et al. “Improved Monomeric Red, Orange and Yellow Fluorescent Proteins Derived from Discosoma Sp. Red Fluorescent Protein.” Nature Biotechnology 22.12 (2004): 1567-72. Print.

11. Komorowski, Michael, Barbel Finkenstadt, and David Rand. “Using a Single Fluorescent Reporter Gene to Infer Half-Life of Extrinsic Noise and Other Parameters of Gene Expression.” Biophysical Journal 98.12 (2006): n. pag. Print.

12. “Alcohol Metabolism: An Update”. U.S. Department of Health & Human Services. National Institute on Alcohol Abuse and Alcoholism. July 2007. 10 Dec. 2013. <http://pubs.niaaa.nih.gov/publications/AA72/AA72.htm>

13. “How Alcohol Is Metabolized in the Human Body.” HAMS Network. The Harm Reduction Network, n.d. Web. 10 Dec. 2013. <http://hamsnetwork.org/metabolism/>.

14. Pisharath, Harshan, Rhee, Jerry M, Parsons, Michael J. “Targeted ablation of beta cells in the embryonic zebrafish pancreas using E.coli nitroreductase.” Mechanisms of Development. March, 2007. 10.1016.