GENETIC VARIATION AND THE TERATOGENIC EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN DURING CARDIOGENESIS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Genetic Variation and the Teratogenic Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin During Cardiogenesis

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Dioxins have historically been known to cause adverse health effects in humans[1]. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), is a byproduct of forest fires, waste incineration, automobile exhaust, cigarette smoke, and other combustion processes[2]. While TCDD is a known teratogen, it has also been linked to lowered sperm counts in male rats[3], cleft palate formation during embryogenesis[4], and kidney blockage[2]. To better understand the teratogenic effects of dioxin, we studied its effect on cardiogenesis. By collecting embryonic hearts from various mouse strains, we investigated potential links between prenatal TCDD exposure and the expression of major genes involved in cardiogenesis. Embryonic hearts were dissected from mice that were prenatally exposed to 0, 1, or 100 ng/kg per day of dioxin. Preliminary data shows significant strain-dependent differences in expression of master regulator genes, such as *GATA4*. We also see delayed expression of other downstream genes involved in heart development that have been known to cause congenital heart defects.

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NOMENCLATURE

TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
Dioxin	2,3,7,8-Tetrachlorodibenzo-p-dioxin

CHAPTER I

INTRODUCTION

Dioxins have been known to cause malformations during embryogenesis. Particularly, 2,3,7,8-tetrachlorodibenzo-p-dioxin, is a potent dioxin commonly found in the environment from combustion processes in industrial facilities. In many locations, these combustion plants are common which leaves humans, especially pregnant mothers, vulnerable to dioxin's potent effects.

TCDD's potent response is mediated by interactions with aryl hydrocarbon receptor (AHR). Normally AHR stays in the cytoplasm in a complex with several proteins, including heat-shock protein 90. However, when TCDD binds to the AHR, it disassociates from the complex and travels to the nucleus. There the AHR-TCDD complex binds to AHR nuclear translocator (ARNT). The active complex then binds to dioxin response elements within DNA.[6]

Cardiogenesis

Since pregnant mothers are highly susceptible to toxicants in the environment, and specifically teratogens, this study focused on pregnant mothers exposed to TCDD. The heart is one of the first organs to develop and can be used to determine toxicity early. TCDD has been known to interact with the mechanisms necessary for development. In an organ such as the heart, this can cause major implications for future development and can cause complications later on in life. In a previous experiment, hearts from chicks were observed after treating with TCDD. Ten out of the thirteen observed TCDD exposed hearts had enlarged right and left ventricles, thickened ventricular septum, and thinner left ventricular walls with increased trabeculation.

Furthermore, AHR and ARNT expression was found in the myocytes of developing atrioventricular canal, outflow tract, and atrial and ventricular septa. This indicated that the developing myocardium and cardiac septa are targets for TCDD-induced toxicity.[7]

Many genes involved in cardiogenesis were quantified in this study. Those genes include: *Nkx 2-5 ORF, Hand1, Hand2, Gata4, Tbx3, Tbx5, Tbx20, GAPDH, β-actin, Pitx2,* and *Mef2c*. These genes were chosen based on their critical roles in developing hearts, a summary of their affects are shown in Figure 1. *Nkx2-5* encodes a homeobox-containing transcription factor. Mutations in the gene can lead to atrial septal with atrial ventricular conduction defect. *Hand1* and *Hand2* are part of the helix-loop-helix transcription factors. Both *Hand1* and *Hand2* create proteins that work complementarily to create the right ventricle and aortic arch arteries.[8]

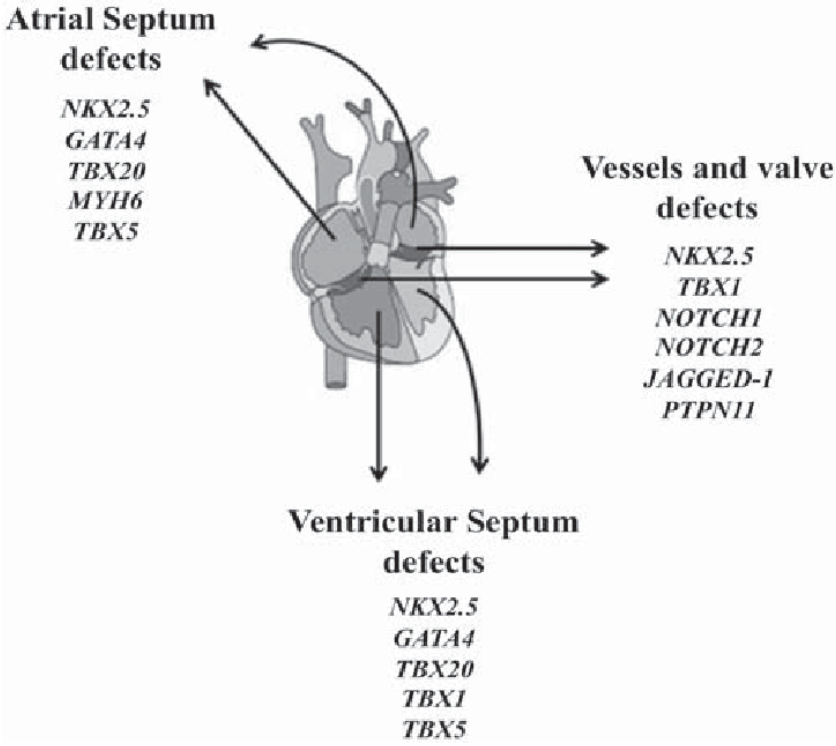

Figure 1. Segmented genes based on defect location.[9]

Gata4 is thought to regulate genes involved in myocardial differentiation and function. Mutations in this gene have been implicated in cardiac septal defects. Along with *Tbx5*, it binds to cardiac super-enhancers and promotes cardiomyocyte gene expression and downregulates endocardial gene expression.[10] *Tbx3*, *Tbx5*, and *Tbx20* are all part of the T-box family, which are classified based on their common DNA binding domain. T-box genes maintain transcription factors involved in the regulation of embryogenesis. However, *Tbx5* and *Tbx20* are more implicated in heart defects. *Tbx20* is critical for heart formation where the gene can lead to defects in septation, valvulogenesis, and cardiomyopathy.[8] *GAPDH* and *β-actin* are found in all cells and respectively help create energy and motility for the cell. *Pitx2* creates a protein involved in the development of abdominal organs. *Mef2c* is a transcription activator in humans and binds to the MEF2 element in regulatory regions of muscles, which can control cardiac morphogenesis and myogenesis. The genes described above are critical in some aspects of cardiogenesis. Through this experiment, information will be garnered that will help determine TCDD's affect on genes related to cardiogenesis. This information may then be related back to human exposure to TCDD and how pregnant woman may be affected. *Gata4* and *Nkx2-5* are of particular importance in this study due to their regulation of downstream genes. Figure 2 demonstrates a few of these regulating pathways.

Along with genes critical for heart formation, two other genes, *Cyp1a1* and *Cyp1b1,* were also analyzed to determine their role in TCDD exposure. *Cyp1a1* and *Cyp1b1* are monooxygenases that help catalyze many reactions related to drug metabolism.[8] If these two genes are upregulated in developing hearts treated with TCDD, then they may have a role in TCDD metabolism, which may be a topic of further study.

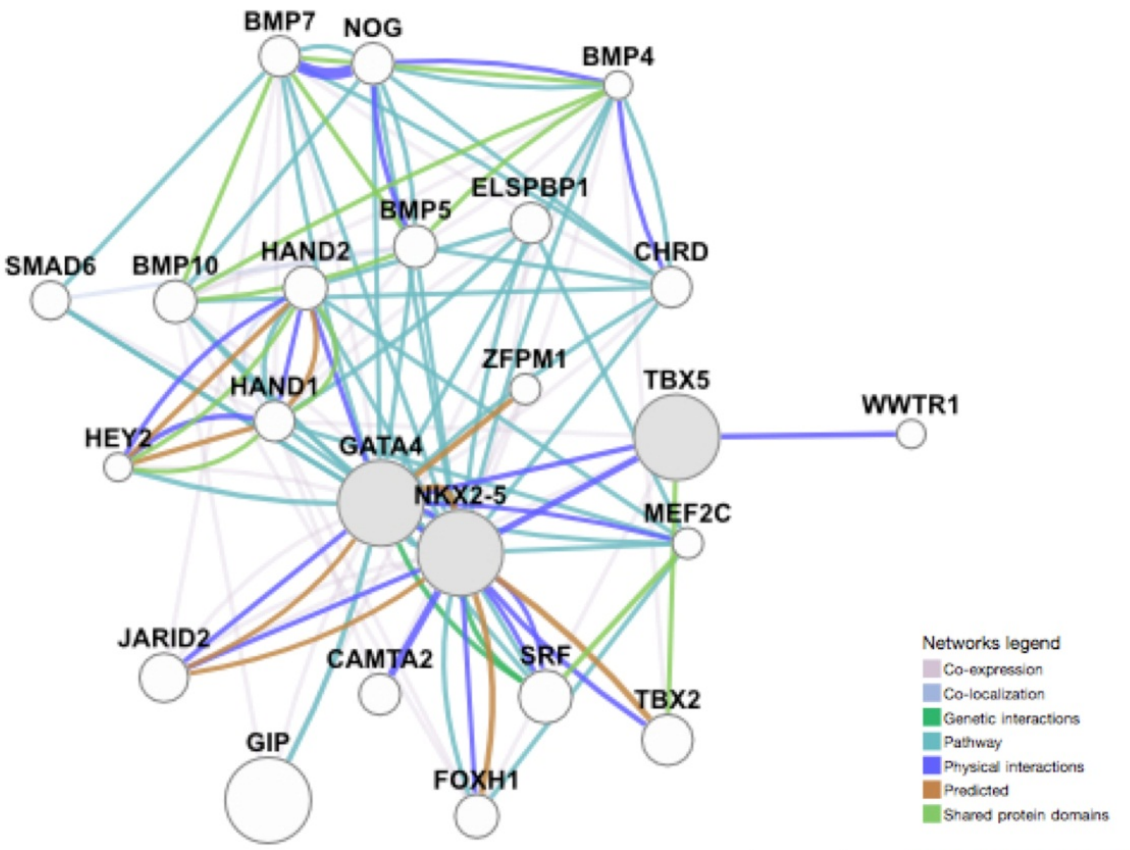

Figure 2: *Gata4* and *Nkx2-5* regulating cascade pathways.[11]

Mouse Models

Mouse models are used due to their genetic diversity, which can reflect that of humans. The embryos collected during this study were from mouse strains that span a variety of families, and included A/J, NOD/ShiltJ, NZO/HiltJ, and BALB/cJ, CC019, CC041, DBA/1J, 129S1/SvlmJ, CBA/J, FVB/NJ, and BXD40. These strains were selected based on their genetic background and their ability to mimic the genetic variance found within the general human population. For instance, A/J have a genetic mutation that causes their hind limbs to under develop. As a result, these mice move slowly and represent individuals who exercise very little.

NZO/HiltJs are severely obese and can be used to study both obesity and Type 2 Diabetes. While other mice, such as FVB/NJ and BALB/cJ demonstrate the average human individual.

CHAPTER II

METHODS

Embryonic heart RNA was collected from an experiment at Texas A&M University that used TCDD treated mice to determine the relationship between dioxin exposure and developing embryos. The mice were chosen based on strain fecundity and variations in genetic traits. In the study, pregnant mothers were dosed with 0, 1, or 100 ng/kg/day of dioxin in peanut butter for the first ten days of pregnancy. Initially the study also included 10 ng/kg and 50 ng/kg dosed mice, but the results were not different from those obtained from the 1 ng/kg dosed mice.

On the first and tenth day of pregnancy, the mice were weighed and given an MRI for body composition. Based on these results, the mice were divided into groups based on weight and were dosed accordingly. Each day, except day ten, the mice were given peanut butter containing a specified dose of TCDD in the morning. On the tenth day of pregnancy, the mice were dosed through oragastric gavage and dissected to confirm pregnancy and collect various organs, such as the uterus.

Using a Leica Light Microscope, the uterus was separated into placentas and embryos. The placentas were collected in a separate tube while the embryonic hearts were removed from the embryos and placed in tubes containing RNA later. Any abnormalities were kept in a separate tube and cited within the research notes.

The embryonic hearts were then treated to isolate the RNA from the hearts. The collected RNA was then run through a Cytation Imaging Reader to determine the amounts of RNA collected. These values were not sufficient to follow the normalization protocol in order to make

cDNA, however the light cycler at the end of the procedure does not require exact concentration amounts.

cDNA Plate Preparation

Since it was discovered in the previous experiment that the quantities of RNA were not enough to follow the normalized method for cDNA preparation, this step was skipped in order to use all RNA available. As a result, 10µL of RNA was used in each well of 96-well skirted plates. From this method, a reference plate was made along with fourteen plates containing samples. These plates were inserted into the Eppendorf epMotion 5075 robot. Using a Qiagen QuantiTect Reverse Transcription Kit, the robot combined quantities of gDNA wipeout buffer, quantiscript reverse transcriptase, quantiscript RT buffer, and RT primer mix. The gDNA wipeout buffer removes any contaminate genomic DNA, while the other components efficiently reverse transcribe the RNA into cDNA. Thirteen plates were made using this method.

qPCR Plate Preparation

qPCR was utilized to detect specific DNA sequences and quantify these sequences. For this project, cDNA plates prepared in the previous steps were used to prepare a qPCR plate inside of an Eppendorf epMotion 5075 robot. The cDNA plates provided the samples and then reagents were measured for creating the new plate. The reagents used were SYBR Green, specific DNA primers, and PCR grade water. SYBR Green works as a DNA or RNA stain. Primers serve as a starting point for DNA synthesis. By selecting what primers to use, certain DNA strands could be replicated. PCR grade water must be used to prevent contamination as the robot formulates the correct concentrations for the Master Mix. These reagents were added to

three individual 2 mL Eppendorf tubes. A fourth 2 mL tube was left empty to perform as the Master Mix tube, a concentration-specific mixture of the reagents combined by the robot. Once all reagents were prepared and placed into the robot, along with the cDNA plate, pipettes, pipette tips, and a qPCR plate, the robot could initiate its application. To create the qPCR plate, the robot used various pipettes as it transferred samples and reagents from the cDNA plate to an empty qPCR plate.

LightCycler

Following qPCR, the qPCR plate was sealed then vortexed and centrifuged to ensure a clear reading when placed into the LightCycler. The LightCycler detects expression of genes from the fluorescence of the SYBR Green. An early logarithmic curve means a higher quantity of gene expression. The results are then compared to a normalized figure of expression per gene. Any qPCR plates that had abnormal readings, were rerun by making another qPCR plate with the same gene. This was to confirm the abnormal readings or correct readings that were inaccurate.

CHAPTER III

RESULTS

Through the SYBR Green, it was discovered that certain plates had high gene expression, while others were nonexistent. Lowered gene expression is concerning in plates, due to its implication to cardiogenesis. Those genes that lack expression allude to cardiogenesis slowing and not forming the chambers of the heart in correct sequence. Severe complications could even lead to a lack of conductance in a fully formed heart.

Currently, only 3-7 mice are used for the results listed below. Performing qPCR on the remaining samples will increase the number of mice per strain and solidify the preliminary data from the study. Data from more mice strains will also be obtained to observe how TCDD affects them as a result of their genetic background. Gene expression for the remaining genes in the study will be quantified as well.

Additionally, the 1ng/kg dioxin dosed mice were removed from the results of the study because there was no marked differences in the values of gene expression.

Lowered Gene Expression

Preliminary results indicate down regulation of the *Nkx2-5* gene. When the results from the mice strains are pooled together, a marked decrease in *Nkx2-5* gene expression can be seen from the control mice to the mice dosed with dioxin. These results are shown in Figure 3. When looking at specific strains of mice, the decrease in *Nkx2-5* is accentuated. This is especially significant in the A/J strain of mice. There is also a trend in down regulation for BALB/cJ, FVB/NJ, and C57BL/6J.

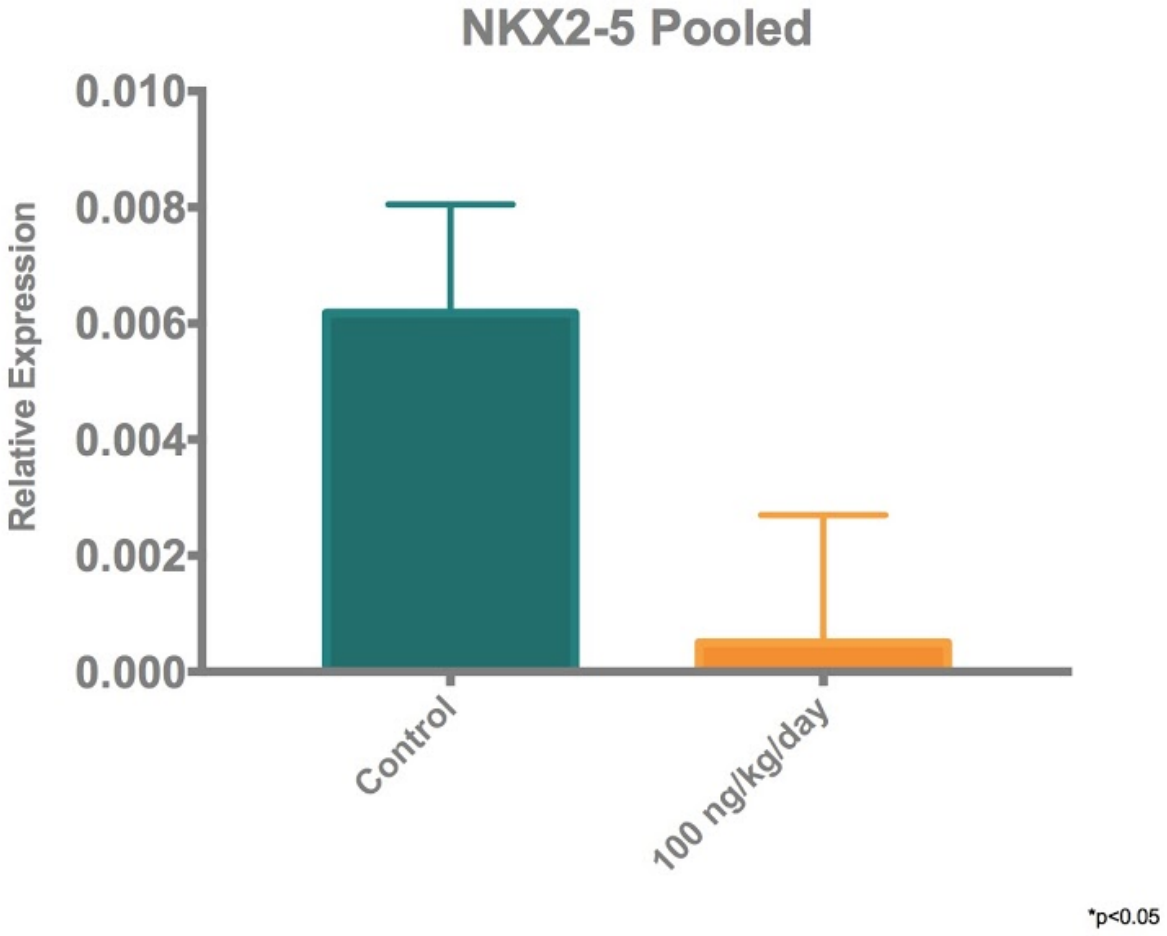

Figure 3: TCDD effect on combined mice strains genetic expression of *Nkx2-5*.

There was little to no change in BXD40 strain of mice. Figure 4 summarizes the findings.

This significant decrease in *Nkx2-5* is concerning. Since *Nkx2-5* is a regulator of many pathways that affect the heart's development, this means that these pathways will also be less activated. This could impair valve and vessel formation along with septal formation of both the ventricles and atriums. Many of these fetuses will not survive to birth. Those that do will be born with congenital heart malformations, such as a hole in the heart. In humans, these children would need to be operated on or they will face many problems performing life-sustaining functions.

Overall the data suggests that dioxin impairs cardiogenesis through downregulating the expression of the gene *Nkx2-5*, which is of paramount importance.

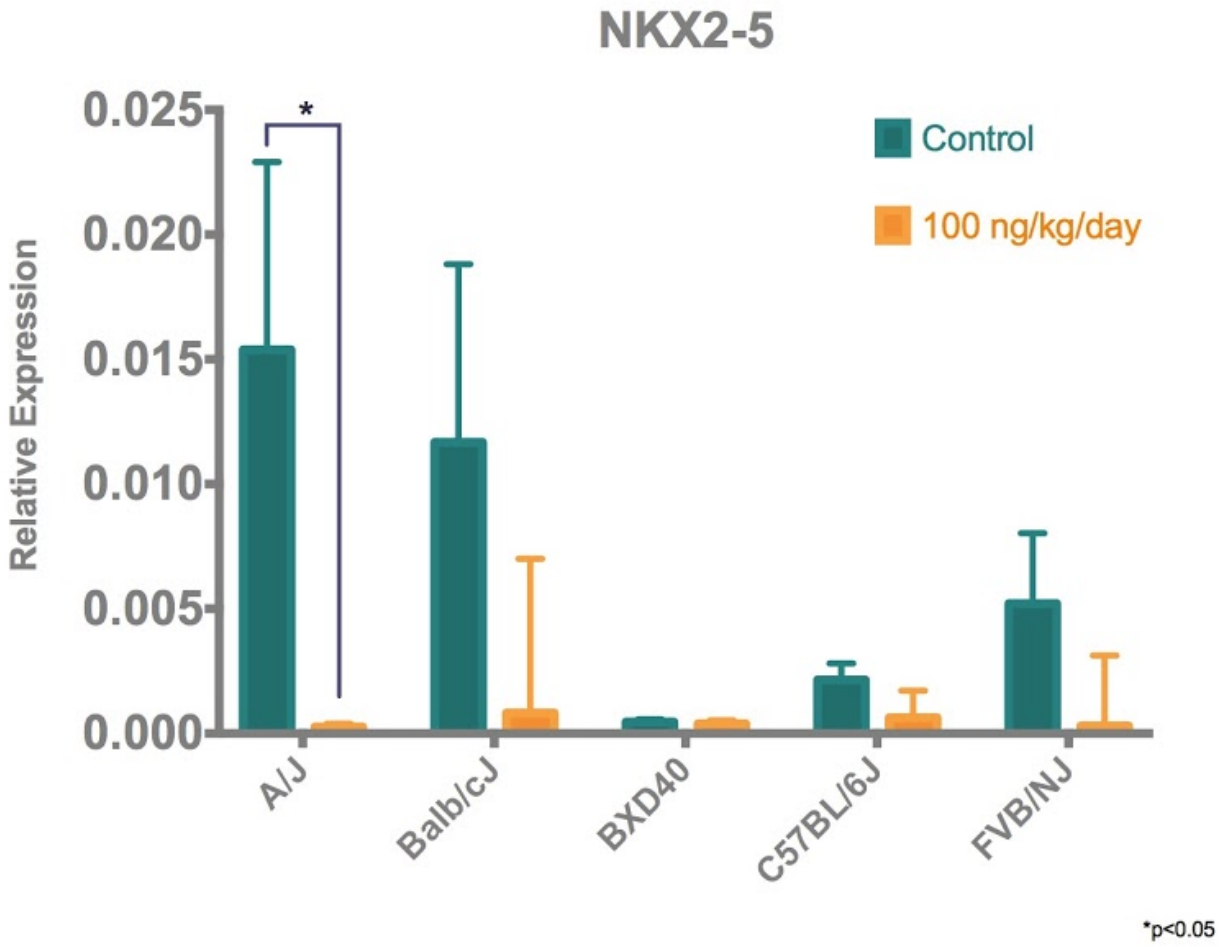

Figure 4: Interstrain variability of *Nkx2-5* expression from TCDD exposure.

Higher Gene Expression

An unexpected result was the increase in *Gata4* expression in some of the mice strains sampled. When pooled together, the results of upregulation are almost negligible as shown in Figure 5. There is only a slight increase in gene expression from the control mice to those dosed with 100ng/kg. However, when the data is separated out into the various strains, as shown in Figure 6, it can be observed that certain strains show upregulation in the *Gata4* gene.

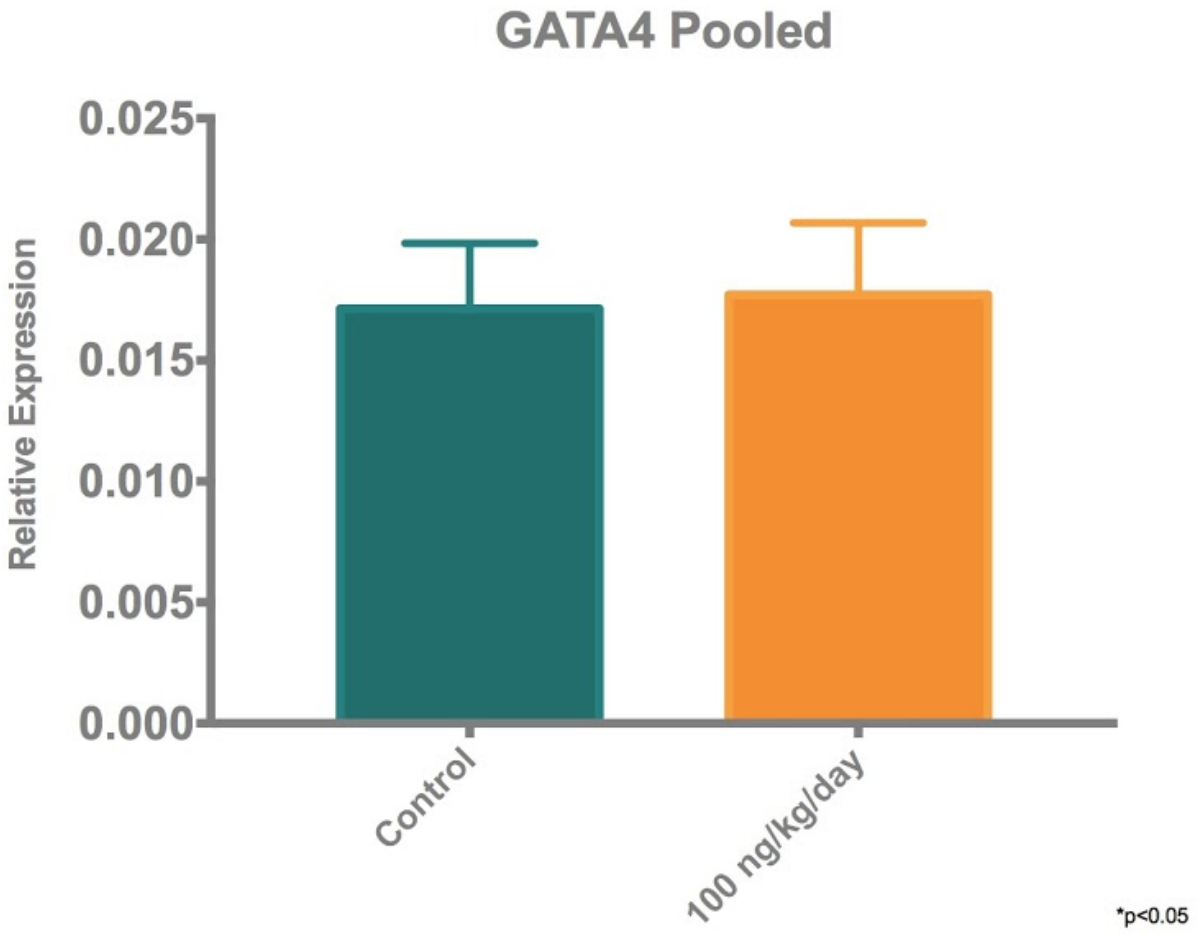

Figure 5: TCDD effect on combined mice strains genetic expression of *Gata4*.

The strains A/J, BXD40, FVB/NJ, and NOD/ShiLtJ followed this trend in upregulation. However, the strains BALB/cJ and C57BL/6J followed the expected outcome of a downregulation in *Gata4* to correspond with the trend for *Nkx2-5*.

While it is uncertain why this upregulation occurs in certain mouse strains, there are a few ideas. One of these ideas is that TCDD may slow down cardiogenesis and embryonic development in general. The expression of the other genes in this study may be able to help glean more information on cardiogenesis depression.

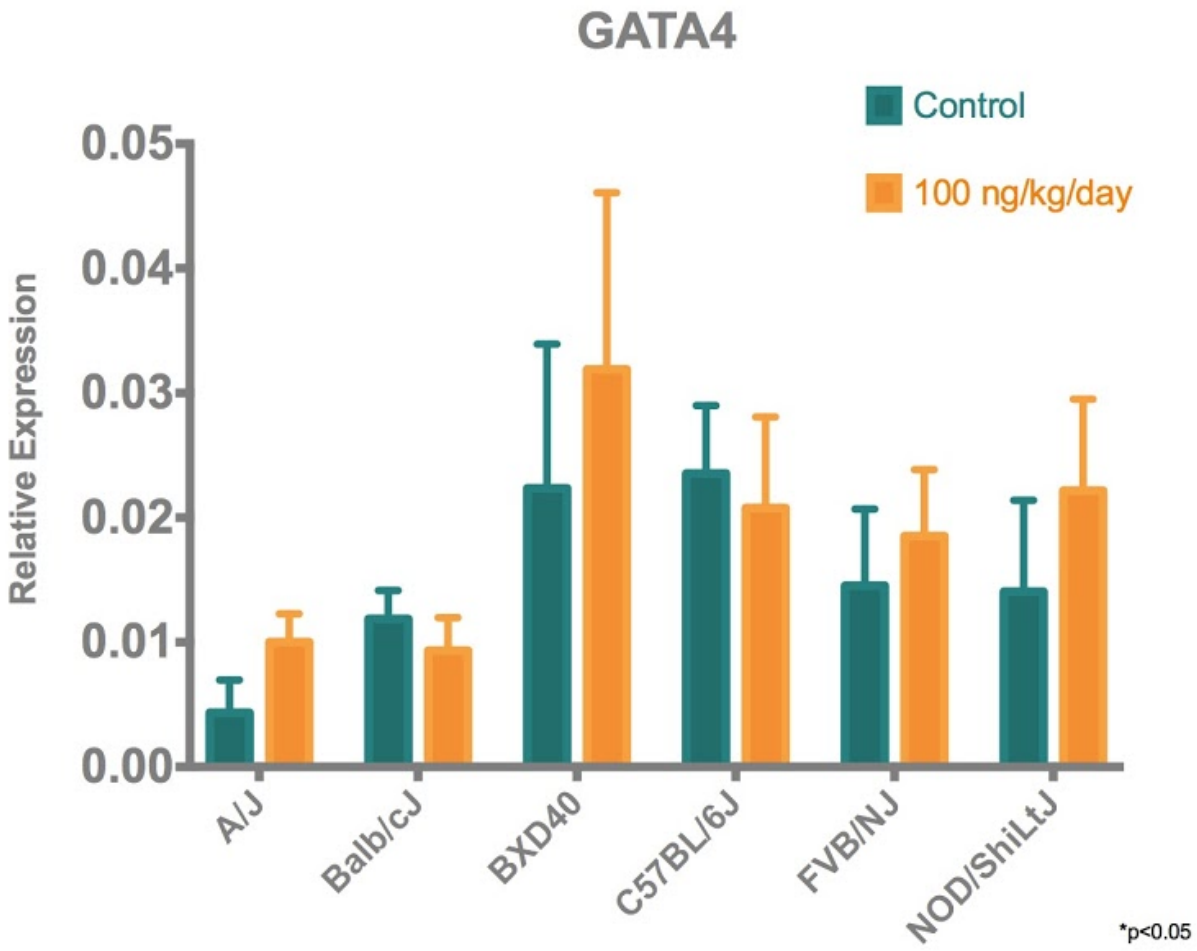

Figure 6: Interstrain variability of *Gata4* expression from TCDD exposure.

CHAPTER IV

CONCLUSION

The findings suggest that TCDD has slowed the progression of cardiogenesis for some strains of mice. This is contingent on the mice strain's ability to with is expected to cause septal and conductance defects in the embryos. This will cause the embryo to no longer be viable or if it is viable it will be left with many congenital heart issues that will have to be treated later on. Additionally, the slowed development, as shown by the *Gata4* upregulation, will cause the embryo to develop more slowly exposing it to more toxicants during development. Many further processes in development may also progress slowly or fail to form. As a result, the baby that is born may be born full term, but will have the characteristics of a premature baby.

Since humans are exposed to dioxin in small amounts over time, pregnant mothers in industrial towns may be at an increased risk. The results from this study suggest that human fetuses may be affected by TCDD by the reduction of *Nkx2-5* gene and a slower progression of development. This may then lead to various developmental problems once the baby is born.

Future experimentation could replicate the study using 20 pregnant mice per strain per dioxin category. Due to the time restraint on the original dioxin study, there was a limit on the number of mice that were dosed with dioxin. The experiment was limited by how many mice were available and how quickly mice would mate. Additionally, future experimentation can determine gene expression in embryonic formation at different stages of development. Once the heart forms, the pregnant mother can be dissected to excise the developing embryo during various time periods of expected heart development point. This study can test how the heart is

affected at different stages of its formation and determine whether heart formation is indeed slowed down by TCDD.

With additional studies, a greater understanding of the affects of TCDD on humans can be determined. Mouse models will help the community become aware of the environmental affects of chemical pollution. The mechanisms behind these pollutants can then be discovered and treatments can be created.

REFERENCES

1. Manuwald U,Garrido MV, Berger J, Manz A, Baur X. Mortality study of chemical workers exposed to dioxins: follow-up 23 years after chemical plant closure. *Occup Environ Med.* 2012;69:636-642.

2. Bryant P, Reid L, Schmid J, Buckalew A, Abbott B. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on fetal mouse urinary tract epithelium in vitro. *Toxicol.* 2001;162:23-24.

3. Hurst C, Abbott B, Schmid J, Birnbaum L. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Disrupts Early Morphogenetic Events That Form the Lower Reproductive Tract in Female Rat Fetuses. *Toxicol Sci.* 2002;65:87–98.

4. Gao L, Yin J, Wu W. Long non-coding RNA H19-mediated mouse cleft palate induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Exp Ther Med.* 2016;11:2355-2360.

5. Armitage J, Ginevan M, Hewitt A, Ross J, Watkins D, Soloman K. Environmental fate and dietary exposures of humans to TCDD as a result of the spraying of Agent Orange in upland forests of Vietnam. *Sci Total Environ.* 2015;506:621-630.

6. Jacobs H, Dennefeld C, Feret B, et al. Retinoic Acid Drives Aryl Hydrocarbon Receptor Expression and Is Instrumental to Dioxin-Induced Toxicity during Palate Development. *Environ Health and Perspect.* 2011;119(11):1590-1595. doi:10.1289/ehp.1003075.

7. Walker MK, Pollenz RS, Smith SM. Expression of the Aryl Hydrocarbon Receptor (AhR) and AhR Nuclear Translocator during Chick Cardiogenesis Is Consistent with 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Heart Defects. *Toxicol Appl Pharmacol*. 1997;143(2):407-419. doi:10.1006/taap.1996.8068.

8. Database GCHG. GeneCards®: The Human Gene Database. GeneCards - Human Genes | Gene Database | Gene Search. http://www.genecards.org/. Accessed February 18, 2018.

9. Vecoli C, Pulignani S, Foffa I, Andreassi M. Congenital Heart Disease: The Crossroads of Genetics, Epigenetics and Environment. *Curr. Genom*. 2014;15(5):390-399. doi:10.2174/1389202915666140716175634.

10. Ang Y-S, Rivas RN, Ribeiro AJ, et al. Disease Model of GATA4 Mutation Reveals Transcription Factor Cooperativity in Human Cardiogenesis. *Cell*. 2016;167(7). doi:10.1016/j.cell.2016.11.033.

11. Fahed A, Nemer G. Genetic Causes of Syndromic and Non-Syndromic Congenital Heart Disease. *Mutations in Human Genetic Disease*. October 2012. doi:10.5772/48477.