THE EFFECT OF CADMIUM ON CHICKEN EMBRYO HEART DEVELOPMENT

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effect of Cadmium on Chicken Embryo Heart Development

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This study aims to elucidate the effect of cadmium (Cd) on chicken embryo heart development. Cd is a hazardous chemical found commonly in cigarette smoke and in food sources due to environmental contaminants. Cd is known to bioaccumulate in organ tissues to cause carcinogenic effects. Our study investigates the effect of Cd exposure during early gestation on embryonic heart development. Phenotypic results show an increasingly hyperplastic myocardial wall associated with increasing $CdCl_2$ dosage and earlier exposure. This semester, we will be using staining through apoptotic markers to determine the mechanism of transport of zinc and cadmium through the embryo. Biochemical analyses and assays will be conducted to further inquire the biological and molecular processes that result in the observed phenotypic changes associated with $CdCl_2$ exposure. We hope to elucidate the mechanism of Cd cardiotoxicity in embryonic development to contribute to identifying susceptibility and treatments for Cd exposure in utero.

ACKNOWLEDGMENTS

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Nicole DePadova would also like to recognize her parents for their constant love and support as well as all the great friends at Texas A&M who have encouraged her to reach her full potential.

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NOMENCLATURE

Cd	Cadmium
Zn	Zinc
HH	Hamburger-Hamilton
HE	Hematoxylin and eosin
qPCR	Quantitative polymerase chain reaction
ICP-MS	Inductively coupled plasma mass spectrometry
PBS	Phosphate buffered saline
DEPC	Diethyl pyrocarbonate
IHC	Immunohistochemistry
LD50	Lethal dose 50
DORV	Double outlet right ventricle
PTA	Persistent truncus arteriosus
OA	Overriding aorta
VSD	Ventricular septal defect
ED50	Effective dose 50

CHAPTER I

INTRODUCTION

As determined by the Center for Disease Control (CDC), Cadmium (Cd) is the 7th highest priority substance heavy metal. [1] Cd is commonly found in phosphate fertilizers, nickel-Cd batteries, plastic stabilizers, pigments, cigarette smoke, some chemical wastes and contaminated foods.[2, 3, 4]

Cd Exposure Routes

Humans can be exposed to Cd in a variety of ways including through inhalation (5-50%), ingestion of contaminated food (1-10%) and a very insignificant amount through skin contact.[5] Exposure via inhalation can occur through inhalation of cigarette smoke; approximately 0.1-0.2 mg of Cd is inhaled per cigarette smoked.[3,6] After absorption, Cd bioaccumulates throughout the body.[5] Within kidney tissue, the half-life of Cd is between 6-38 years and is between 4-19 years within human liver tissue.[5] For all other human tissues, the half-life of Cd ranges from 9-47 years.[5] Another common route of exposure for humans is through ingestion of contaminated dietary products such as mushrooms, seafood, vegetables (up to 150 μg Cd/kg), fruits (up to 50 μg Cd/kg) and previously contaminated animal kidneys.[4]

Effect of Cd Toxicity in Humans

Cadmium is recognized as a carcinogen in humans, and exposure to Cd increases the risk of being diagnosed with breast and renal cancer.[7,8,9] Furthermore, chronic Cd poisoning was shown to increase the risk for liver damage, pneumonia, gastrointestinal, cardiovascular and renal diseases.[9] During pregnancy, Cd exposure has been associated with restricted growth in the fetus.[10]

Effect of Cd Toxicity in Animals

Studies on animal Cd exposure have shown Cd accumulation in fetal tissue when the mother is exposed during pregnancy ultimately causing fetal growth restriction.[11] The bulk of Cd research that has been conducted has focused on exposure in young and adult vertebrates. Studies have shown Cd exposure causes cardiovascular inflammation in rats, thinner ventricular walls in chickens and an overall reduced heart weight in chicken embryos.[12, 13] Morphological abnormalities were also observed in animals exposed to Cd in later developmental stages of their lives.[14] Other studies concerning Cd exposure in chickens have shown that abnormalities in the cephalic, liver and reproductive systems occurred as a result.[14, 15, 16] However, many of the effects and mechanisms on the early stages of development are unexplored. Figure 1 summarizes the routes of entry of Cd in human and animal tissue along with the most common effects it has.

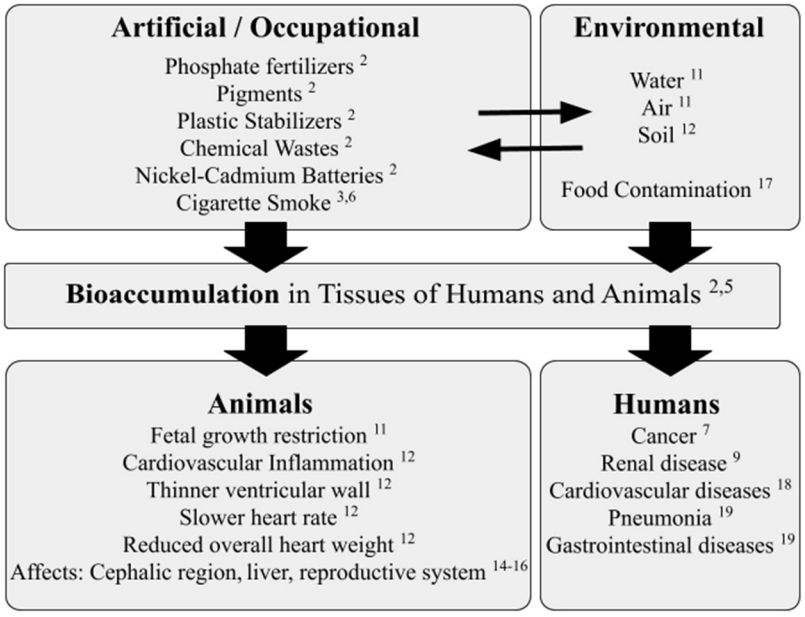

Figure 1. Common routes of entry in Cd toxicity

Objective and Hypothesis

The objective of our research is to elucidate the teratogenic effects of cadmium exposure during early stages of chicken embryonic myocardium development. Our hypothesis is that, due to the extensive myocardium development that takes place between HH 13-16 and HH 27-28, we expect to see an increase in heart abnormalities with increasing Cd dosage compared to the chicken saline control.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

To assess the effect of cadmium injection on embryonic heart development, varying concentrations of cadmium chloride, either 10 μM, 50 μM, 100 μM or a saline control, were injected into Gallus gallus single-comb White Leghorn eggs. The eggs were divided into two groups and injected with cadmium chloride ($CdCl_2$) solution at either Hamburger-Hamilton (HH) Stage 13-16 (2 days after fertilization) or HH Stage 27-28 (5 days after fertilization), times when significant myocardial development takes place. They were incubated in a rocking egg incubator at 39 °C ± 0.5 °C until the time of collection. All of the eggs were collected at either five or nine days after fertilization depending on technique being performed. Cadmium concentrations in the yolk and amniotic fluid were measured at the time of sample collection. The eggs were then processed by utilizing histological techniques such as Paraffin embedding and HE staining in order to view each heart under a high-power microscope. A further overview of the experimental design is provided below in Figure 2.

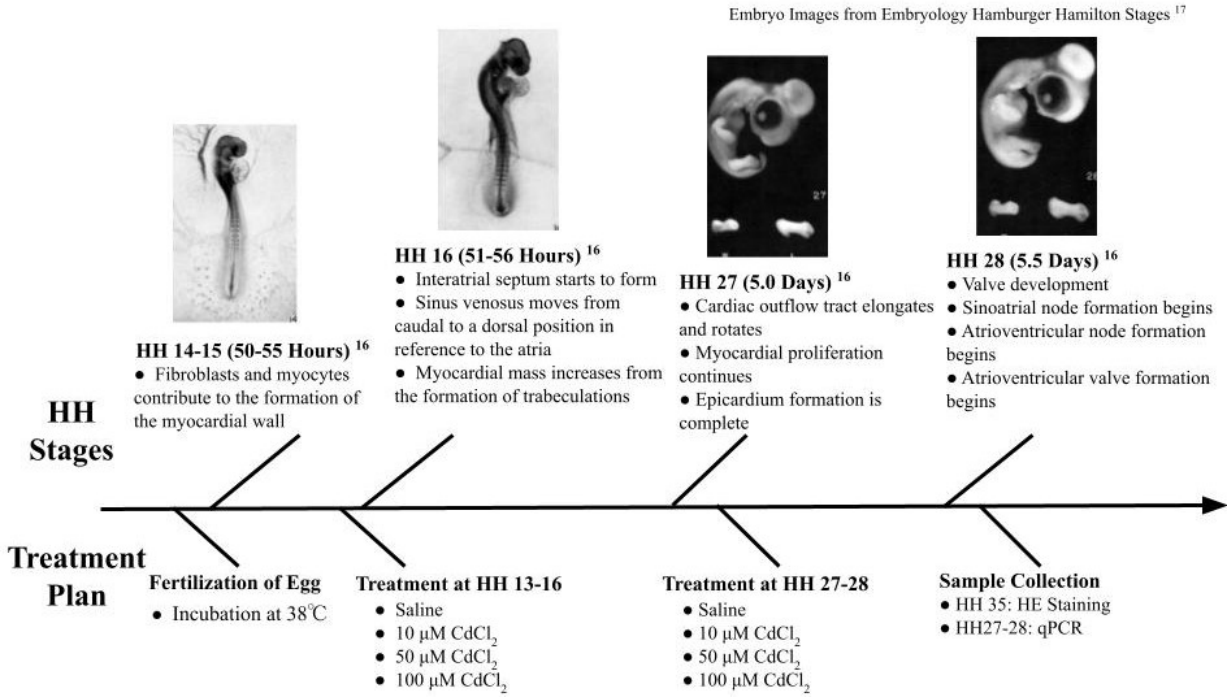

Figure 2. Sample injection and collection timeline for histologic analysis

Cd Solution Preparation and In-Ovo Injection of Solutions

For the saline control, chicken saline solution was prepared using NaCl (8.47 g), $CaCl_2$ (0.79 g), $MgCl_2$ (0.162 g), KCl (0.4 g). Each egg received 100 mL of either saline or cadmium chloride. The amount of cadmium chloride was calculated using Equation 1. The weight of each individual egg was measured to determine the $CdCl_2$ concentration necessary, and the solution was diluted to 100 mL with saline. A sample calculation for a 100 µM injection is provided below in Table 1.

Sample Calculation: $V_1 = M_2V_2 / M_1$ (Eq. 1)

Table 1. Sample injection calculation for $CdCl_2$

Concentration Needed	Volume	Formula	Solution
100 µM	10 mL	V1=[1X10^-4/1000mLX10mL]/[0.1/1000mL]	Use 10 µL of 0.1 M in 10 mL of saline

Eggs were divided between HH 13-16 and HH 27-28 injection groups with an even number in each treatment group. Injection protocol was based on a previous study by Ko, et al. 2013.[20] To inject the calculated solution through the inner membrane, forceps were used to make a hole approximately ± 0.5 cm^2 at the top of each egg. The treatment amount was injected via 100 μM pipet under vent hood UV light, and the hole was covered with gauze tape before being placed back into the rocking egg incubator.

Sample Collection

Embryos utilized for qPCR and ICP-MS were injected at day 2 and collected at day 5 or injected at day 5 and collected at day 9. Embryos for histology were injected at either day 2 or day 5 and collected at day 9. For collection, the gauze tape was removed and a larger hole in the top of the shell was created. Survival rate and fertilization status were recorded. Amniotic fluid and yolk were collected for histology studies and placed into separate 1.5 mL Eppendorf tubes. For qPCR and Cd and Zn concentration studies, the yolk, amniotic fluid, atrium, ventricles, and embryo were each individually collected. The embryo was placed into a petri dish with PBS to wash (or PBS + DEPC for qPCR embryos) and relocated to a well plate 70% full of 10% formalin. Pictures of the embryonic limbs were taken while in the PBS solution. After two days in the formalin solution, each embryo was removed and the lower portion of the body from the liver down was removed with microscissors. Additionally, the head and neck were removed. To embed the samples for tissue sectioning, they were incubated in paraffin and embedded in plastic cassettes using paraffin wax. In samples utilized for qPCR, the heart was dissected out and split into left and right atria before being stored in Trizol solution.

Histology Techniques

Hematoxylin and Eosin Staining

Samples for staining were cooled in an ice bath, sectioned using a Microtome at 5 µM thickness, and placed onto slides for staining. To begin HE staining, all slides were dried and incubated overnight at 60 °C. Xie Lab's Hematoxylin & Eosin Staining protocol was followed to stain each slide as follows: incubation at 60 ℃ (minimum 40 mins), xylene two times (5 mins each), 100% EtOH two times (2 min each), 90% EtOH (2 mins), 70% EtOH (2 mins), wash in running tap water (2 mins), Hematoxylin (1.5 mins), wash in running tap water (5 mins), Eosin-Y (1 min), wash in running tap water (2 mins), dry overnight, and apply a mounting coverslip glass.

Qualitative Analysis Visual Identification Hyperplastic Myocardium

All stained tissue samples were analyzed for a visual phenotype of hyperplastic myocardium, the experimental condition, using two criteria. Hyperplastic myocardium was determined to be present when (1) the sample had a greater amount of trabeculations when compared to the saline control or (2) had less open space in the ventricle than overall myocardium area. If the hyperplastic myocardium phenotype was present in a specific ventricle for a sample, it was indicated as a 1. The normal phenotype indicated as 0 for statistical analysis.

Quantitative Analysis Myocardium Tissue Coverage

Three model samples where both valves were visible in a single slide were selected for each day in each group: saline control, 10 µM, 50 µM, 100 µM. Three students each measured the myocardium area of the left and right ventricles for day 2 and day 5 embryos using ImageJ software. Stipulations for where to measure each embryo were verbalized to all students. A ratio

of myocardium tissue percentage was calculated by measuring the entire ventricle area and dividing by the myocardium area (area % = area of myocardium / area of ventricle x 100%).

Quantitative Image Analysis

Image J Protocol

The protocol was adapted from Mr. Jie Wu, a former student of Dr. Zhang, whose previous work used coding to take measurements in this software. The protocol was utilized to measure myocardium tissue coverage for all selected model samples for both day 2 and day 5 embryos.

ImageScope Protocol

This protocol was utilized to measure the ventricular wall. Each student measured five individual embryo sections on a slide for each of 21 embryo samples using a single blinded technique whereby the treatment type was unknown. Eight measurements were taken total, four on the left ventricle and four on the right according to the following predetermined criteria: Measurement 1 is at the root of the ventricular valve. Measurements 2 and 3 were evenly spaced along the ventricle wall. Measurement four was taken at the thickest portion of the ventricle.

Cd and Zn Concentration Measurement

Inductively coupled plasma mass spectrometry (ICP-MS) was performed to analyze the respective concentrations of cadmium and zinc in the embryonic yolk. Eggs were divided into equal groups and injected either at day 2 and collected at day 5 or injected at day 5 and collected at day 9. The injection solution contained either saline control, 10 μM cadmium, 10 μM zinc, 50 μM cadmium or 50 μM zinc. To digest the yolk for analysis, 0.1 grams of yolk was combined with the amount of metal present in either the blank or matrix spike and 400 μL nitric acid. The solution was incubated in a cryogenic vial at 90 °C for three hours and cooled for 20 minutes

before adding an additional 300 µL nitric acid and 300 µL hydrogen peroxide. This was heated for 1 hour at 85 °C. After cooling to room temperature, 1 mL 1% nitric acid was added and the mixture was moved into a 15 mL tube for machine analysis. The calibration standards for the machine were as follows: Zn: 1 ppb, 5 ppb, 10 ppb, 100 ppb, 200 ppb, Cd: 0.01 ppb, 0.05 ppb, 0.1 ppb, 0.5 ppb, 1 ppb. Standards were created by taking a designated amount of solution for each respective sample and diluting to 50 mL with 1% nitric acid. Once a pressure of 250 psi and a temperature below 15 °C was reached, the samples were run through the machine and ion concentrations were analyzed.

Quantitative Polymerase Chain Reaction

Quantitative PCR was utilized to identify genotypic changes caused by cadmium injection. Embryos were collected at either day 5 or day 9 based on time of cadmium exposure. Genes significant in either apoptotic processes or cell proliferation were individually tested for, and the samples were standardized utilizing GAPDH (gene-GAPDH). Tissue for extraction was placed in Trizol solution and homogenized before chloroform was added. The sample underwent a series of purifications with buffer and isopropyl alcohol, with intermittent rounds of centrifugation. Purified RNA was converted to cDNA for further analysis by combining RNA template, Ready Script cDNA, and RNAase/DNAase free water in a 0.2 mL tube. The tube was centrifuged and incubated for a total of 40 minutes at increasing temperatures. Diethyl pyrocarbonate (180 mL) was added to the 20 µL cDNA before qPCR was run. For statistical analysis, the fold change was calculated using the formula $2^{-\Delta\Delta CT}$.

Statistics and Statistical Significance

When interpreting results, P-values less than 0.05 were considered statistically significant, and p-values greater than 0.05 but less than 0.1 were considered marginally

significant. Marginal significance is denoted by a ▲, while statistical significance is denoted by a ✱. A logistic model curve fitting was used to determine the lethal dose 50 (LD50) from the survival rates. The statistical significance of the survival rate and visually identified qualitative phenotypes were grouped by treatment day and dosage and analyzed for statistical significance using the Fisher's exact test. Two-sample t-tests comparing the control group and treatment groups were performed to analyze the statistical significance of myocardium area.

CHAPTER III

RESULTS AND DISCUSSION

Effect of Cd on Survival Rate and LD50

As our preliminary study, the objective was to determine the effect of treatment time on survival rate and LD50. Survival rate was determined for each embryo at the time of collection and recorded in Table 2. Fisher's exact test showed a statistically significant decrease in survival rate in HH 13-16 embryos with exposure to increasing concentrations of cadmium when grouped together and compared to the control. Survival rate at day 2 100 µM was significantly decreased at 12.5%, while day 5 exposure embryos showed no significant change in survival rates. This decrease in survival rate in day 2 embryos suggests early exposure to cadmium increases the risk of embryonic death.

Table 2. Survival rate is lower in day 2 treated embryos compared to day 5

Treatment	**Treatment Time**	
	HH 13-16 (Day 2)	**HH 27-28 (Day 5)**
Saline	100% (n=14)	88.89% (n=9)
10 µM	100% (n=10)	93.75% (n=16)
50 µM	90.91% (n=11) *	94.12% (n=17)
100 µM	12.50% (n=8)*	66.67% (n=15)

The lethal dose 50 is the amount of toxin sufficient to skill 50% of the population. A logistic model curve was used to generate the LD50 for day 2 and day 5 embryos. The LD50 in HH 13-16 embryos was 69.179, and the LD50 in HH 27-28 embryos was 98.379 (Figure 3).

Both survival rate and LD50 decreased with early exposure to cadmium. This indicates that early exposure embryos are more sensitive to the effects of cadmium than later exposure embryos.

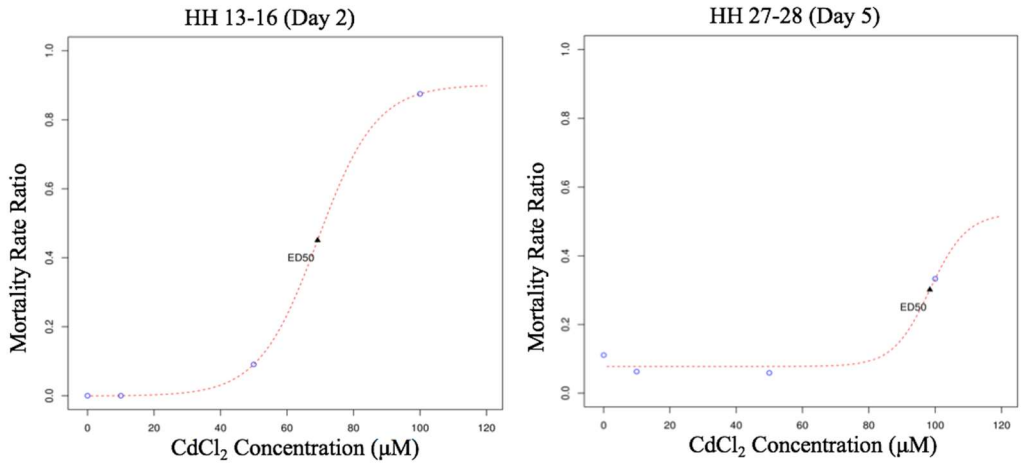

Figure 3. LD50 for early exposure embryos is lower than LD50 in later exposure embryos

Effect of Cd on Hyperplastic Myocardium Phenotype Expression

Determination of ventricular hyperplasia was performed by qualitatively comparing treatment samples to their corresponding controls. Examples of images utilized to diagnose left and right ventricles are seen below in Figure 4. The saline controls at each day are indicative of normal ventricular development, while the $CdCl_2$ treated embryos in Figure 4 were all considered to possess evidence of hyperplasia. Visual identification of the hyperplastic phenotype suggested an increase in frequency of hyperplastic myocardium with earlier exposure and greater Cd dosage.

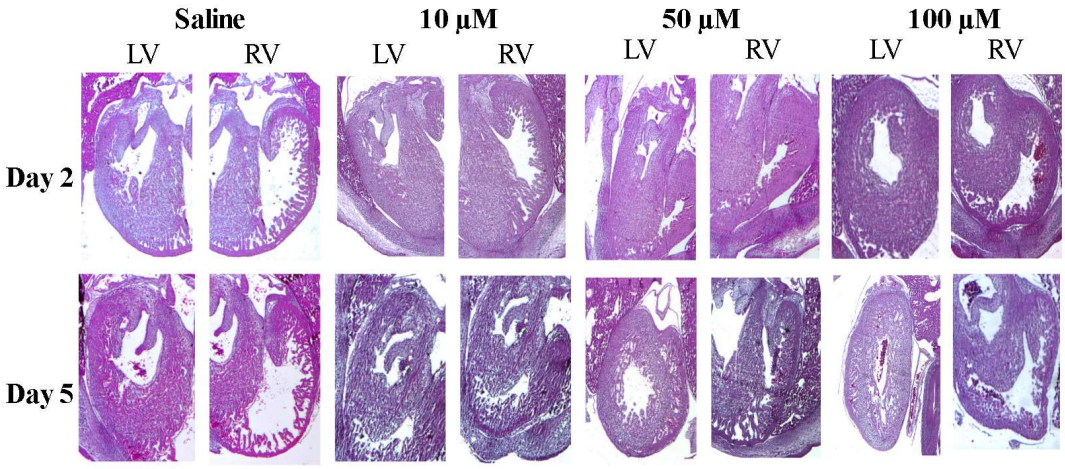

Figure 4. Visualization of hyperplastic myocardium for experimental groups compared to saline control

Effect of Cd on Myocardium Percentage

Myocardium percentages were calculated by both students individually for the left and right ventricles for day 2 and day 5 embryos. In day 2 (Figure 5 a-d), the experimental concentrations grouped together show a statistically significant increase in myocardium percentage for both students in the left and right ventricles. For both students, the day 2 left ventricle myocardium percentages showed a statistically significant increase in response to $CdCl_2$ at 50 µM and 100 µM (p=0.000 [student 1], p=0.000 [student 2]). For day 2 right ventricle, there was a statistically significant increase at 10 µM, 50 µM, and 100 µM. At day 2, the right ventricle showed a greater increase in myocardium percentage from control to experimental groups than the left ventricle for both students. In day 5 right ventricle (Figure 5 e-h) myocardium percentages increased for all three experimental doses of $CdCl_2$ as well as when the 10 µM, 50 µM, and 100 µM were grouped together and compared to the saline control (p= 0.078, 0.0004, 0.017, 0.002 [student 2], p=0.093, 0.001, 0.027, 0.005 [student 3]). In the left ventricle, myocardium % increases at 10 µM (p=0.029 [student 2], p=0.005 [student 3], but then

decreases at higher concentrations for both students. For day 5 left ventricle, when all experimental groups were compared to the control, there was only statistical significance in one student's data set (p=0.012 [student 2], p=0.174 [student 1]). The earlier exposure day 2 samples showed a greater increase in myocardium percentages than day 5. Additionally, the right ventricle is more sensitive to cadmium induced myocardium changes than the left ventricle. With the exception of student 1's day 5 LV data, increased cadmium levels lead to an increase in myocardium % when compared to the saline control. This suggests that Cd exposure-response is exacerbated by increasing dosage and exposure at earlier developmental stages.

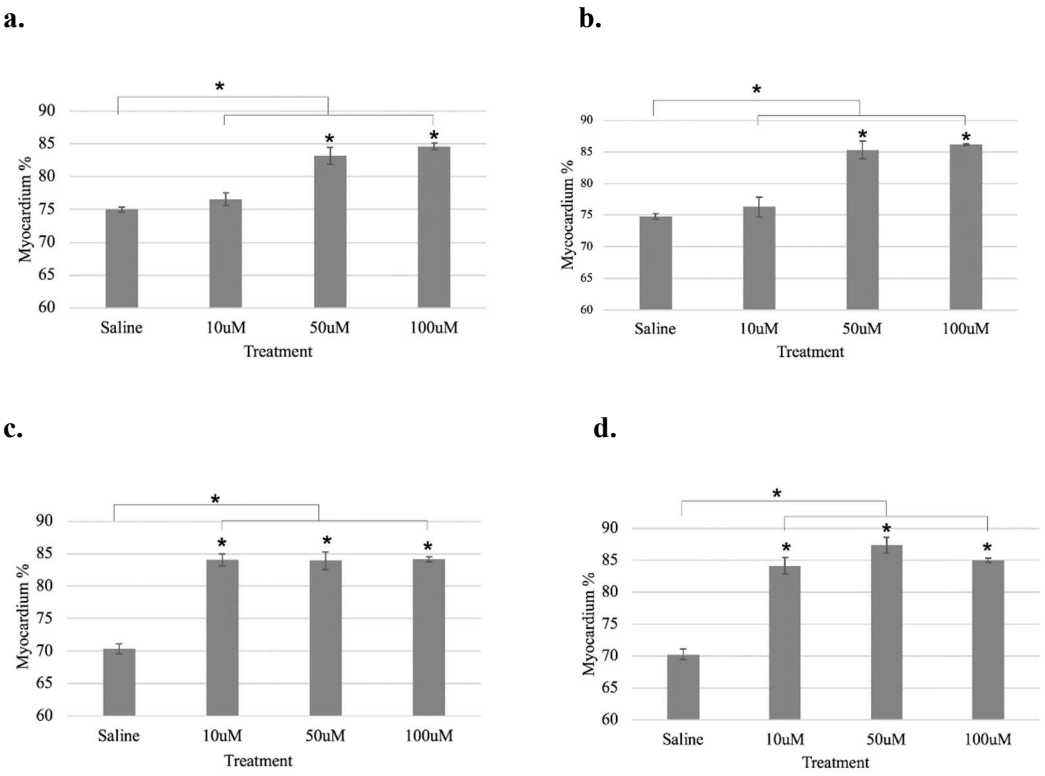

Figure 5 a-d. Increasing concentrations of cadmium at earlier exposure times increases overall myocardium percentage a. Day 2 LV myocardium % measurements by student 1 b. Day 2 LV myocardium % measurements by student 2 c. Day 2 RV myocardium % measurements by student 1 d. Day 2 RV myocardium % measurements by student 2

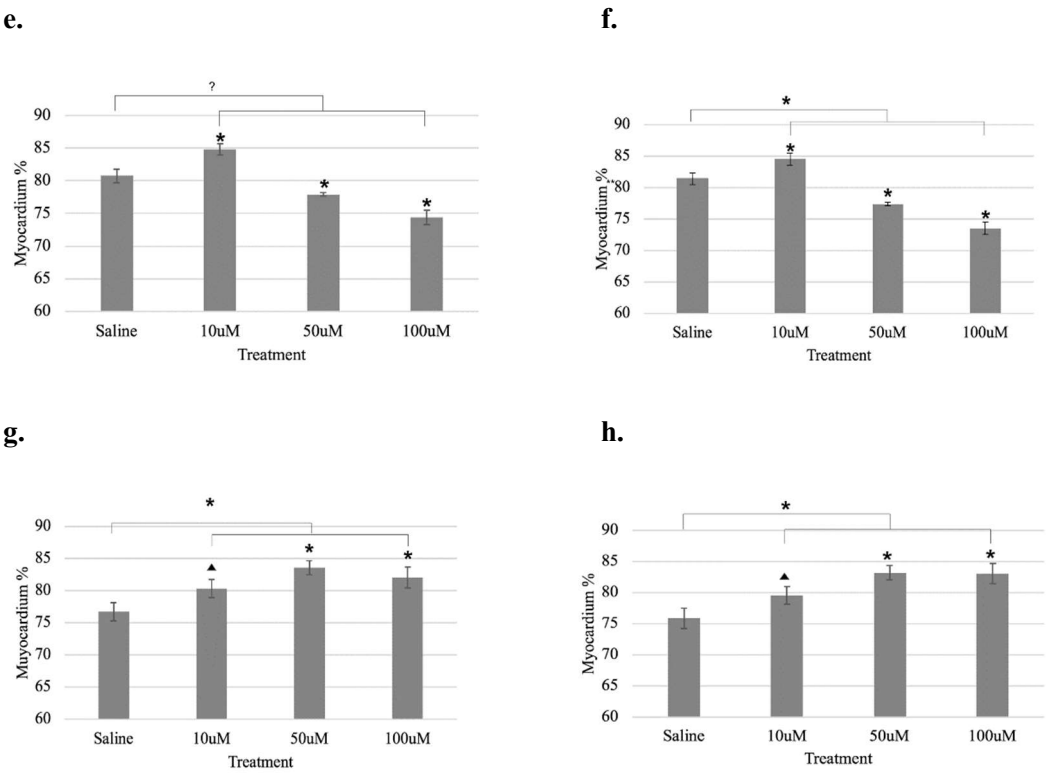

Figure 5 e-h. Increasing concentrations of cadmium at earlier exposure times increases overall myocardium percentage e. Day 5 LV myocardium % measurements by student 1 f. Day 5 LV myocardium % measurements by student 2 g. Day 5 RV myocardium % measurements by student 1 h. Day 5 RV myocardium % measurements by student 2

Cd and Zn Uptake

The objective of ICP-MS was to observe how concentrations of Cd and Zn change based on length of exposure to each metal. We hypothesized that increased exposure to cadmium would result in increased uptake of cadmium into the embryo and therefore decreased concentrations in the yolk. In day 2 injection of cadmium (Figure 6 a), there was a statistically significant increase ($p=0.0109$) in cadmium concentration in the yolk at 10 µM in the day 9 embryos when compared to day 5. Day 2 zinc injection did not show any significant relationship between time of exposure and zinc concentrations in the yolk, though a slight decrease in zinc concentration in the treated groups compared to saline was present (Figure 6 b). Likewise, day 5

injection day 9 collection of cadmium in Figure 6 c did not show any significant uptake of Cd into the embryo. However, increasing concentrations of cadmium in day 5 injection day 9 collection embryos (Figure 6 d) lead to a decreased amount of zinc in the yolk when compared to saline at 50 µM (p=0.013) and 100 µM (p=0.0046). There was also a significant decrease in zinc levels between 10 µM and 50 µM (p=0.0152) and between 10 µM and 100 µM (p=0.0056). As Cd concentrations increase, it appears more zinc is taken up into the embryo for use. Cadmium is hypothesized to share a transport pathway with zinc[21] and may provide insight into cadmium's mechanism of toxicity.

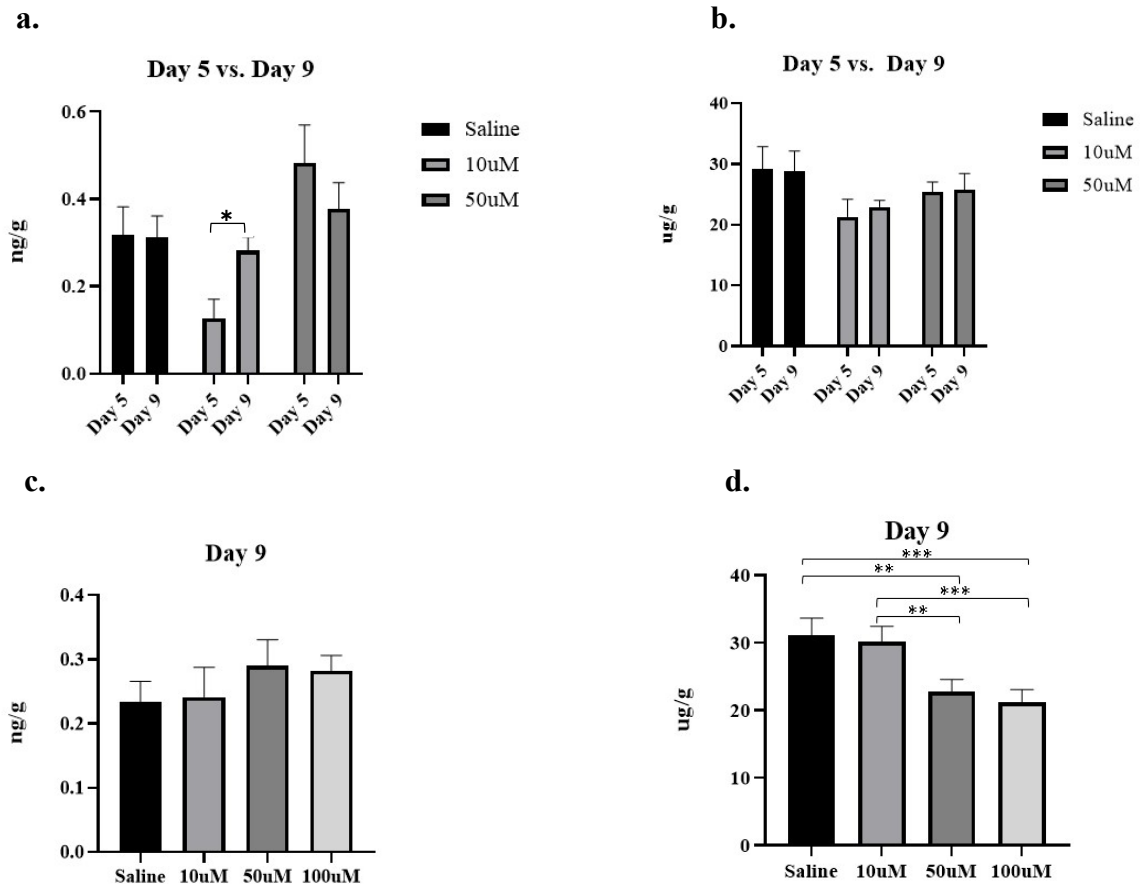

Figure 6 a-d. Comparison of cadmium and zinc concentrations in day 5 and day 9 collection groups a. Day 2 injection with cadmium, collection at day 5 or day 9 b. Day 2 injection with zinc, collection at day 5 or day 9 c. Day 5 injection with cadmium, collection at day 9 d. Day 5 injection with zinc, collection at day 9

Effect of Cd on Ventricle Wall Thickness

As a continuation of our single-blinded study, four students from the Xie lab individually conducted measurements on the ventricular walls of all samples using ImageScope software in a single-blinded manner, shown in Figure 7 a-d.

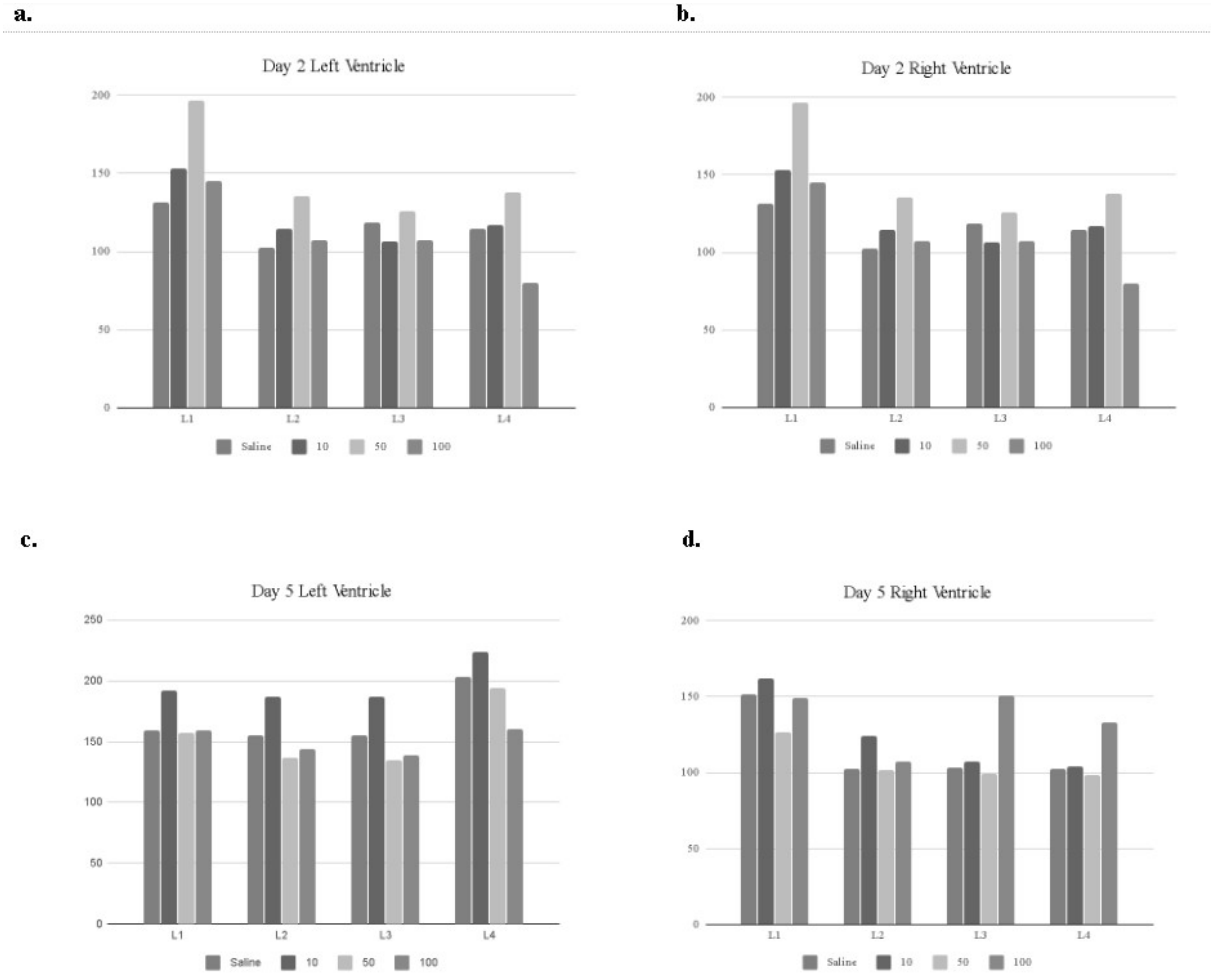

Figure 7 a-d. Individual ventricle wall thickness measurements show minimal statistical significance due to a large intrapersonal variability. Each L_1, L_2, etc. represents a different student who performed the measurements a. Ventricle wall thickness measurements day 2 left ventricle b. Ventricle wall thickness measurements day 2 right ventricle c. Ventricle wall thickness measurements day 5 left ventricle d. Ventricle wall thickness measurements day 5 right ventricle

This study design introduced sufficient intrapersonal variability that made it difficult to deduce any clear trends. Utilizing the ImageScope software, it was difficult to uphold standard

criteria between students. Ventricular wall thickness showed an overall increase in response to cadmium treatment. However, the amount of fluctuation between measurements made it difficult to derive any sort of conclusion from the data. A statistical analysis was performed on our data collected from ventricle measurements of both chicken and mice exposed to Cd. The data showed no significant difference in ventricular thickness between Cd-treated samples and the control in either animal. If this type of study were to be repeated in the future, stricter and more standardized protocol would have to be implemented and consistently monitored for each student.

Effect of Cd on Occurrence of Heart Defects

Our study aimed to observe the presence of four heart defects: double outlet right ventricle (DORV), persistent truncus arteriosus (PTA), overriding aorta (OA), and ventricular septal defects (VSD). Defects were observed in day 2 and day 5 injection samples that were collected on day 9. No OA or VSD were present in any of the hearts. A pairwise Fisher's Exact test was performed on the data in Table 3, and no statistical significance between cadmium exposure and incidence of heart defects was detected at day 2 embryos or day 5 embryos.

Table 3. Heart defects present in cadmium treated embryos showed no statistical significance based on dose or exposure period

a.

Day 2 Treatments	DORV		PTA		OFT defect (DORV or PTA)	
	Positive (+)	Normal	Positive (+)	Normal	Positive (+)	Normal
Saline	1	7	0	7	1	7
10 uM	2	8	1	8	3	8
50 uM	2	7	1	7	3	7
100 uM	0	1	0	1	0	1

b.

Day 5 Treatments	DORV		PTA		OFT defect (DORV or PTA)	
	Positive (+)	Normal	Positive (+)	Normal	Positive (+)	Normal
Saline	3	5	0	5	3	7
10 uM	3	7	1	7	4	8
50 uM	1	5	0	5	1	7
100 uM	1	4	0	4	1	1

Notes. Positive indicates the presence of the heart defect, while normal indicates the lack of a heart defect a. Heart defects in day 2 treated embryos b. Heart defects in day 5 treated embryos

Effect of Cd on Occurrence of Limb Defects

Qualitative visualization of limbs, seen in Table 4, showed no absence of limbs associated with cadmium chloride treatment in the early exposure group or the later exposure group.

Table 4. Limb defect analysis on both collection groups showed no observable absence of limbs with Cd treatment

a.

	Forelimb		Hind Limb	
D2 at D9	**Absent**	**Present**	**Absent**	**Present**
Saline	0	5	0	5
10 uM	0	9	0	9
50 uM	0	10	0	10
100 uM	0	1	0	1

b.

	Forelimb		Hind Limb	
D5 at D9	**Absent**	**Present**	**Absent**	**Present**
Saline	0	8	0	8
10 uM	0	15	0	15
50 uM	0	15	0	15
100 uM	0	8	0	8

Notes. a. Limb analysis for embryos injected at day 2 and collected at day 9 b. Limb analysis for embryos injected at day 5 and collected at day 9

qPCR Analysis

Genes integral to proliferation and apoptosis were identified and analyzed. HH 27-28 ventricles from HH 13-16 $CdCl_2$ treated samples showed a marginal downregulation in Cyclin E (cyc E) in the 50 μM dose (Figure 8). Cyc-e is a proliferative gene involved in regulating cyclin dependent kinase and other protein kinase activity. HH 27-28 ventricles from $CdCl_2$ treated samples showed significant downregulation in Apo2L at and marginal downregulation of Fas at dose 10 μM (Figure 9). Apo2L is involved in cytokine activity metal ion binding which induces apoptosis and is involved in the immune response. Fas is a transmembrane signaling receptor

activity involved in the apoptotic process as well. Ventricles from HH 27-28 $CdCl_2$ treated samples showed marginal downregulation in Casp9 and Fas at dose 50 µM (Figure 9). Casp9 activates caspases responsible for apoptosis initiation by activating cell death proteins or inactivating survival proteins. Overall, the qPCR results indicate that increasing concentrations of cadmium are associated with an overall downregulation of apoptotic genes.

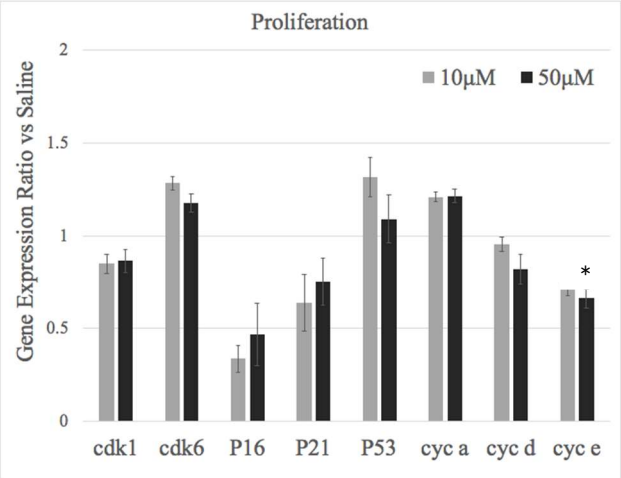

Figure 8. Proliferative gene results show marginal down regulation in Cyclin E in day 5 treated ventricles in the 50 µM dose. 1 is considered the 'baseline/saline', an increase above 1: upregulation, a decrease below 1: downregulation

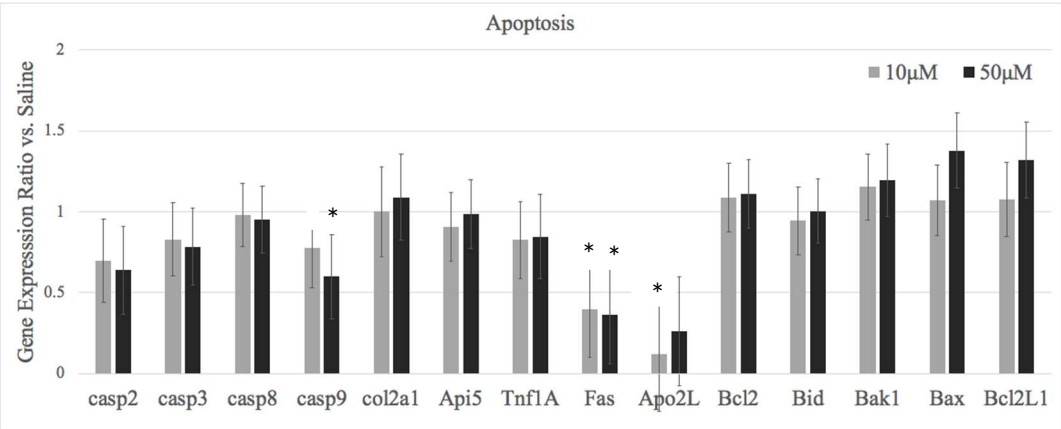

Figure 9. Genes associated with apoptotic processes show marginal down regulation in Casp9 and Fas at dose 50 µM at day 5 treated ventricles and a significant downregulation in Apo2L. 1 is considered the 'baseline/saline', an increase above 1: upregulation, a decrease below 1: downregulation

CHAPTER IV

CONCLUSION

Analysis

Overall, this experiment showed that both the LD50 and survival rate decrease with an earlier exposure to Cd, and an earlier/larger dose of Cd causes an increase in both myocardium percentage and risk of hyperplastic myocardium development.

The effective dose 50 (ED50) for the LV of day 2 and day 5 $CdCl_2$ exposures were determined to be 23.703 μM and 48.033 μM, respectively through the fitting of a logistic model curve. The ED50 results indicate that early embryonic development has an increased sensitivity to Cd exposure compared to the later stages of development. This also suggests that the RV is more sensitive than the LV to $CdCl_2$ treatment in both day 2 and day 5 exposures.

Day 2 100 μM $CdCl_2$ treatment resulted in high incidence of embryonic death. Day 2 treated embryos had a decreased survival rate, thus proving that an earlier exposure to Cd increases the risk of death. Hyperplastic myocardium phenotype visual identification showed an increase in myocardium in both day 2 and day 5 $CdCl_2$ treatments but more significantly in day 2 compared to the control. This indicates that an earlier exposure to Cd increases the likelihood of a hyperplastic myocardium phenotype. Both the LV and RV in day 2 treated samples showed a dose dependent response regarding myocardium percentage increase. Day 5 treated Cd samples also showed an increase in myocardium percentage in the RV but is not indicative of a dose-dependent response. Day 5 10 μM $CdCl_2$ shows an increase in left ventricle myocardium %; however, this trend reverses for the LV because the myocardium percentage decreases as Cd dosage is increased to 50 μM, 100 μM, or grouped together. Day 2 samples showed a greater

myocardium percentage increase indicating that a greater Cd-exposure response was seen in these samples over day 5. Overall, this suggests that an increased Cd dosage causes a greater myocardium percentage increase. Our findings also suggest that early Cd exposure affects myocardium development in both a dose-dependent and time sensitive manner.

Implications of Research

Overall, our research indicates that exposure to Cd toxicity during the early stages of myocardium development results in: (1) a decreased survival rate, (2) increased hyperplastic phenotypes within the ventricle, (3) a greater percentage of myocardium tissue and (4) downregulation of apoptotic genes (Fas, Apo2L,casp9) in the ventricles. These results suggest that Cd exposure in early stages of fetal development has an effect on chicken embryo myocardium development. This study also indicated a relationship between zinc and cadmium uptake in the developing embryo. However, the origin of the Cd responses and mechanisms still remain unknown.

Future Directions

Due to the unforeseen events surrounding COVID-19 virus in spring 2020, completion of TUNEL staining data and H3S10 staining data were unavailable at the time of publication of this URS thesis. In the future, we hope to attain these results in order to form an enhanced picture of the effects of cadmium on the apoptotic and proliferative processes of cardiomyocytes. TUNEL staining aims to detect the presence of apoptotic cardiomyocytes, while H3S10 aims to identify cells going through active cell division. These results will provide further information regarding cadmium's effect on myocardium development. Additionally, the lab will continue to refine protocol regarding visual analysis of hyperplastic phenotypes to ensure a more consistent result. This will also provide better insight into myocardium development in early exposure embryos.

Our study has developed a more comprehensive knowledge of the role of cadmium at early developmental stages as well as the role of cadmium on cardiomyocyte development. It has also provided additional analysis on the relationship between zinc and cadmium in the developing embryo. We anticipate future studies on this topic will further elucidate cadmium's mechanisms of toxicity in order to better understand its role in embryonic development. Understanding cadmium's role in embryonic development will be critical to implementing solutions to prevent cadmium induced toxicity and will be crucial in mitigating cadmium exposure in developing embryos.

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