EFFECTS OF CHRONIC CADMIUM EXPOSURE ON THE
CARDIOVASCULAR SYSTEM

by

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Abstract

**Background:** As the prevalence of cardiovascular disease remains high, there is a developing focus on risk factors beyond those that are modifiable and, in particular, those ubiquitous within our environment. An abundance of epidemiological studies has shown an association between cadmium exposure and cardiovascular diseases including hypertension, atherosclerosis, myocardial infarction and heart failure. However, there are clear knowledge gaps in the literature, and the present study sought to better define the effects of chronic cadmium exposure on the cardiovascular system. **Methods:** In this study, female and male mice were exposed to 5 ppm of cadmium chloride (CdCl2) via drinking water for 8 weeks. During this period, blood pressure was measured and serial echocardiography was performed to measure for changes in morphology and contractile function of the left ventricle. Mice were then sacrificed and all hearts were analyzed for the development of hypertrophy, some were subjected to ischemia reperfusion injury, and others deemed for molecular analysis. **Results:** CdCl2 exposure did not alter body weight, food consumption, water consumption, blood pressure and expression of renal eNOS and Akt in mice subjected to these exposure conditions. However, CdCl2 exposure was associated with a statistically significant reduction in interventricular septum thickness at end-systole, ejection fraction and fractional shortening in the hearts of male mice. **Conclusion:** Most notably, these results suggest that chronic cadmium exposure causes a sex-dependent reduction in left ventricular function and septal thickness. These findings support cadmium as a potential driver of cardiovascular disease.

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CHAPTER 1

1. Introduction to Cardiovascular Physiology

The cardiovascular system brings together a combination of specialized cells, tissues, and organs that function together to maintain adequate circulation and, in turn, homeostasis throughout the body. It includes the heart, blood, blood vessels and lymphatic system (Iaizzo, 2015).

1.1. Blood

Blood is liquid tissue that facilitates the transportation of dissolved gases, nutrients, waste products and signaling molecules as well as the regulation of pH, temperature, and pressure (Iaizzo, 2015). Specifically, blood consist of three formed elements suspended in plasma; erythrocytes, leukocytes, and platelets (Iaizzo, 2015). Erythrocytes contain hemoglobin, a protein molecule that binds and releases molecular oxygen (O₂) and carbon dioxide (CO₂) during respiration (Iaizzo, 2015). Leukocytes are cells of the immune system that are formed within the bone marrow and aid in the protection against foreign agents and disease (Iaizzo, 2015). Platelets are cellular fragments that are important in the clotting process (Iaizzo, 2015).

1.2. Blood Vessels

Blood vessels include the network of arteries, veins and capillaries which enclose the unidirectional flow of blood (Iaizzo, 2015). Major vasculature includes the superior
vena cava and inferior vena cava that delivers deoxygenated blood to the heart, the pulmonary artery that delivers deoxygenated blood from the heart to the lungs, the pulmonary vein that delivers oxygenated blood from the lungs to the heart, and the aorta that branches into the arteries that delivers oxygenated blood throughout the rest of the body (Iaizzo, 2015). These arteries branch into arterioles, and arterioles branch into capillaries (Iaizzo, 2015). The capillaries form capillary beds, where this increased surface area allows for greater gas exchange with cells undergoing cellular respiration (Iaizzo, 2015). The capillaries then reassemble to form venules, and venules reassemble to form veins carrying deoxygenated blood back to the heart and lungs (Iaizzo, 2015).

### 1.3. Myocardium

The human heart is located in the thoracic mediastinum and is centered left of the body’s midline (Iaizzo, 2015). It is comprised of four chambers; right atria, right ventricle, left atria, and left ventricle, with the atria directed superior to the ventricles (Iaizzo, 2015). On the surface of the heart, the deep coronary sulcus differentiates the atria from the ventricles and the anterior interventricular sulcus differentiates the left side from right side of the heart (Iaizzo, 2015). The relative wall thickness of each chamber is directly proportional to the generated force required to pump blood through that chamber. This translates to thicker walls on the left side of the heart relative the right side as well as thicker ventricular walls than atrial walls throughout (Iaizzo, 2015). Valves are connective tissue structures that open and close in response to pressure changes within the heart and help to control the unidirectional flow of blood. The four valves in the heart
include the tricuspid valve; pulmonary valve, bicuspid valve and the aortic valve (Hill & Iaizzo, 2015).

The heart pumps blood through the synchronized contraction of its four chambers. The chambers are in systole during contraction and diastole during relaxation (Laske et al., 2015). The contraction of a cardiac muscle exerts a force on the blood contained within each chamber and the resulting increase in pressure pushes blood through valves and into adjacent vasculature or chambers of lower pressure (Laske et al., 2015). During atrial contraction, the increasing pressure, ranging from 0-8 mmHg in the right atrium and 1-10 mmHg in the left atrium, causes the opening of the tricuspid and bicuspid valves and flow of blood into the right and left ventricles, respectively. While filling with blood, the ventricles are in diastole with pressure ranging from 0-8 mmHg in the right ventricle and 3-12 mmHg in the left ventricle (Iaizzo, 2015). During ventricular systole, the heart is initially in isovolumetric contraction, in which there is no change in ventricular volume (Iaizzo, 2015). This increases the ventricular pressure, ranging from 15-30 mmHg in the right ventricle and 100-140 mmHg in the left ventricle, and it causes the closing of the tricuspid and mitral valves (Iaizzo, 2015). This increase in pressure then causes the pulmonary and aortic valves to open, allowing blood to move from the right and left ventricles flows into the pulmonary artery and into the aorta respectively (Iaizzo, 2015).

The intrinsic conduction system of the heart drives this contraction and is comprised of nodes and branches of autorhythmic fibers (Laske et al., 2015). When a node generates an electrical impulse, it spreads across muscle fibers via gap junctions which are protein channels directly connecting the membranes of adjacent cardiomyocytes (Laske et al., 2015). The electrical impulse originates from the sinoatrial
node (SA node) within the right atrium, and this electrical impulse travels through both atria and causes these chambers to contract (Laske et al., 2015). As this electrical impulse drives the contraction of the atria, it also reaches the interatrial septum and causes the excitation of the atrioventricular node (AV node) and, in turn, the bundle of his (Laske et al., 2015). The bundle of his branches into right and left bundles down the septum, and these branches separate into the Purkinje fibers of the right and left ventricles. The electrical impulse travels through these fibers of the ventricles and causes these chambers to contract slightly after atrial contraction (Laske et al., 2015).

Specifically, synchronized action potentials underlie these traveling electrical impulses. Action potentials are changes in the membrane potential of a cell and occur through the depolarization and repolarization of its membrane (Laske et al., 2015). At resting state, the membrane potential of a contractile cell within the heart is -90 mV (Laske et al., 2015). Depolarization occurs when an action potential from a neighboring cell activates the opening of voltage-gated sodium channels in the membrane of an adjacent cell (Laske et al., 2015). This causes the influx of positive Na+ ions and the membrane potential to become more positive (Laske et al., 2015). The voltage-gated sodium channels quickly inactivate (Laske et al., 2015). Voltage-gated calcium channel begin to open, leading to an influx of positive Ca2+ ions. Repolarization occurs when the voltage-gated calcium channels close and voltage-gated potassium channels open to move positive K+ ions out of the cell, driving the membrane potential back to -90 mV (Laske et al., 2015).

As described above, the depolarization of the membrane causes the movement of Ca2+ ions into the contractile cell during the plateau phase (Barnett, 2015). At this
moment, the influx of extracellular Ca2+ also causes the dihydropyridine (DHP) receptors to undergo a conformational change in the transverse tubules of the sarcolemma (Barnett, 2015). This initiates a release of Ca2+ ions from the sarcoplasmic reticulum into the cytoplasm (Barnett, 2015). These Ca2+ ions bind to troponin to regulate the interaction between actin and myosin which are the contractile fibers within the heart (Barnett, 2015). Actin is referred to as the thin filament and myosin is referred to as the thick filament (Barnett, 2015). Based on the sliding filament theory, contraction occurs when these filaments slide to overlap one another and, in turn, shorten the length of the sarcomere (Barnett, 2015). The length-tension relationship states that the shortening of the sarcomere creates myocyte tension. (Barnett, 2015). At rest, troponin and tropomyosin cover the active site on actin, which prevents the binding of the myosin head and, in turn, the interaction of the filaments (Barnett, 2015). During an action potential, the release of free Ca2+ ions in the cytoplasm bind to troponin, causing it to undergo a conformational change and release from actin (Barnett, 2015). This reveals a binding side on actin thus allowing the myosin head to bind (Barnett, 2015). Binding causes myosin to transition from a low-energy configuration after the hydrolyzation of ATP. The release of ADP and inorganic phosphate drives the power stroke which shortens the sarcomere and, in aggregate, causes the muscle to contract (Barnett, 2015). The binding of ATP releases myosin from actin, and with a decline in Ca2+ facilitated by Ca2+ uptake into the sarcoplasmic reticulum and Ca2+ extrusion from the cell, troponin and tropomyosin cover actin until the next action potential (Barnett, 2015).

2. Cardiovascular Disease
Cardiovascular disease (CVD), which was responsible for 853,125 deaths in the United States in 2017 alone, can disrupt this homeostasis (Virani et al., 2020). Cardiovascular disease include hypertension, coronary heart disease, peripheral vascular disease, heart failure, congenital heart disease, and cardiomyopathies. As observed in many of these conditions, CVD can result in endpoints including cardiac hypertrophy, fibrosis, and hypertension. As the leading cause of mortality globally and in the United States, CVD encompasses conditions of the blood vessels and heart (Murphy et al., 2018). Based on a forecasting study conducted in 2011, crude CVD prevalence will be 40.5% by 2030 and direct medical costs will have tripled from 2010 to 2030 (Heidenreich et al., 2011).

2.1 Oxidative Stress

The damage associated with many cardiovascular diseases can attributed to increased generation of reactive oxygen species (ROS), which often drives an imbalance between homeostatic ROS levels and antioxidant systems (da Cunha Martins et al., 2018). Among several others, ROS include superoxide O$_2^-$ and potent oxidant-peroxynitrite (ONOO$^-$) (da Cunha Martins et al., 2018). ROS can induce damage to macromolecules including DNA, protein and lipids (Beevers et al., 2001; da Cunha Martins et al., 2018) Antioxidant systems have evolved to combat this damage, and alterations to these antioxidant systems can include disruptions to the functional group of
antioxidant molecules and binding of essential metal cofactors (da Cunha Martins et al., 2018).

2.2. Hypertension

Hypertension occurs as a result of an imbalance between peripheral vascular resistance and cardiac output while presenting as high blood pressure. Hypertension is caused by a change in several physiological mechanisms including oxidative stress, endothelial dysfunction, catecholamine changes, and alterations to the renin-angiotensin system (Beevers et al., 2001; da Cunha Martins et al., 2018). Specifically, the activation of the sympathetic nervous system causes the release of renin from the kidneys (Paz Ocaranza et al., 2020). Renin is an enzyme that converts angiotensinogen to angiotensin I. Angiotensin I-converting enzyme (ACE) converts angiotensin I to angiotensin II, leading to several intermediate steps that ultimately increase blood pressure (Paz Ocaranza et al., 2020; Santos et al., 2012). An increase in blood pressure beyond homeostatic levels increases the demand of the heart and can ultimately induce incidences of myocardial infarction and/or hypertrophy (Hinderliter et al., 2019).

2.3 Myocardial Hypertrophy

Myocardial hypertrophy is a form of cardiac remodeling that presents as an increase in myocyte size/volume and commonly aggregates into a structural change to the
ventricular walls of the heart. The two types of cardiac hypertrophy, physiological and pathological, are defined by their cause and effect on cardiac function. Pathological hypertrophy can develop from neurohormonal activation, high blood pressure and myocardial injury (Shimizu & Minamino, 2016). In pathological hypertrophy, insufficient angiogenesis leads to hypoxia and subsequent remodeling of the heart. More specifically, this usually manifests as an increase in left ventricle size and is commonly defined as concentric or eccentric hypertrophy. In concentric hypertrophy, sarcomeres are added in parallel and there is a thickening in the left ventricle wall (Shimizu & Minamino, 2016). Whereas in eccentric hypertrophy, sarcomeres are added in series and the left ventricle chamber is dilated (Gjesdal et al., 2011; Shimizu & Minamino, 2016). This reduces the efficiency of cardiac contraction and, consequently, can lead to heart failure. Specifically, concentric hypertrophy leads to diastolic heart failure and eccentric hypertrophy leads to systolic heart failure (Gjesdal et al., 2011; Shimizu & Minamino, 2016). Cardiac hypertrophy is an active area of research, because there remains an incomplete understanding of all the pathways involved.

2.3.1. Calcineurin/ NFAT Signaling

Heightened intracellular calcium levels can activate several calcium dependent pathways in cardiomyocytes, with the calcineurin/ NFAT signaling pathway being one of the most studied and understood in cardiac hypertrophy (Kehat & Molkentin, 2010; Shimizu & Minamino, 2016). Once activated by calcium in the cytoplasm, calcineurin, a serine-threonine phosphatase, dephosphorylates the nuclear factor of activated T cells
transcription factor (NFAT) (Kehat & Molkentin, 2010; Shimizu & Minamino, 2016). This induces a conformational change and rapid translocation of NFAT into the nucleus, where it binds to a NFAT response element on DNA and causes downstream hypertrophic gene expression (Kehat & Molkentin, 2010; Shimizu & Minamino, 2016). As expected, any alternations to homeostatic intracellular calcium, calcineurin levels or NFAT activity may induce pathological hypertrophy, and therefore should be tested when studying pathways associated with cardiac remodeling.

2.4. Cardiac Fibrosis

Cardiac fibrosis is caused by the accumulation of excess extra cellular matrix (ECM) in the tissue of the heart (Ma et al., 2018). Among other components, the ECM contains signaling molecules and a network of different types of collagen aiding in tissue structure and elasticity. (Ma et al., 2018). The components of the ECM are maintained in balance to prevent the aggregation and, ultimately, cardiac remodeling, cardiac impairment, reduced capillary density and electrophysiologic dysfunction (Ma et al., 2018). Cardiac fibrosis occurs in three phases: initiating phase, effective phase and amplification phase (Ma et al., 2018). In short, fibrotic stimuli bind to cardiac fibroblast receptors and initiate the conversion of cardiac fibroblasts to active myofibroblasts (Ma et al., 2018). Cardiac fibrosis decreases cardiac contractility and compliance by stiffening the walls of the heart (Ma et al., 2018).

2.5. Myocardial Ischemia-Reperfusion Injury
Ischemia occurs when there is inadequate blood flow to the heart, often resulting from an occlusion or reduced flow within the associated vasculature. During this period of restricted oxygen and nutrient delivery, anaerobic glycolysis replaces mitochondrial oxidative phosphorylation causing a decrease in pH, an increase in intracellular Ca\textsuperscript{2+} and reduced cardiac contractility (Frank et al., 2012). Immediately following ischemia, the rapid recovery of blood flow, known as reperfusion, reduces injury but also causes some tissue damage due to the increased generation of ROS with the recovery of mitochondrial oxidative phosphorylation. The formation of ROS along with myofibril hypercontractility caused by the rapid recovery of pH and increased calcium, induces injury, cell death and increases infarct size (Frank et al., 2012).

3. Cardioprotective Processes

As described above, both ischemia and reperfusion can induce cell death. Interestingly, there are cardioprotective mechanisms within the heart that oppose this damage and, in turn, reduce infarct size. Cardioprotective processes include those endogenous to the heart as well as those that can be triggered via external factors. Elucidating the mechanisms underlying this protection is an active area of research, especially as researchers and clinicians seek to apply this knowledge to the development of new therapies. In particular, exposing the heart to brief episodes of nonlethal ischemia has been shown to help the heart build resistance to ischemic damage and, as a result, attenuate infarct size (Furchgott, 1996; Sun et al., 2013; Ziolo et al., 2008). This
phenomenon of ischemic preconditioning is thought to occur naturally in the heart and presents as anginal pain in an individual just prior to an ischemic event (Rosenberg et al., 2018). One of the earlier studies into pre-conditioning was conducted in 1986 where researchers discovered that preconditioning the dog heart to four 5-minute ischemic episodes prior to a 40-minute circumflex coronary occlusion period resulted in reduced necrosis compared to the control hearts (Rosenberg et al., 2018). These researchers speculated that the mechanisms of protection induced a slowing of ATP depletion either by reducing ATP utilization or increase ATP production. Additionally, they suggested the protection may result from a decreased accumulation of ischemic catabolites; either by flushing the system of these catabolites during the pre-conditioning or by the reduced demand for anaerobic glycolysis (Rosenberg et al., 2018). Recent research into cardioprotection has discovered that nitric oxide plays a critical role and metallothionein upregulation is thought to confer protection as well. Mimicking this natural conditioning through mechanical and pharmacological intervention has proven to pose considerable limitations, but promising research into the mechanisms and signaling cascades is ongoing.

Interestingly, there is an apparent sex difference in cardioprotection. Prior studies have shown that premenopausal females have lower incidences of CVD and differences in cardioprotective signaling pathways (Murphy et al., 2011). Specifically, research has shown that estrogen plays a role in mediating this sex difference, specifically by increasing eNOS expression and p13K signaling in the female heart (Murphy et al., 2011).
3.1. Nitric Oxide

Nitric Oxide (NO) is a stable, uncharged, and paramagnetic molecule found diffusing throughout our body. The free electron makes nitric oxide reactive with radical molecules and transition metals but relatively unreactive with molecules within the body (Lancaster, 2015). •NO reacts with O₂ to form the nitrogen dioxide radical (•NO₂), which is an oxidant that can react further with nucleophiles (Lancaster, 2015). •NO also reacts with molecular oxygen (O₂) to form nitrite (NO₂⁻), which is regarded as a physiological reservoir of nitric oxide (Lancaster, 2020; Shiva, 2013). Additionally, •NO reacts rapidly with superoxide (O₂⁻) to form the oxidant peroxynitrite (ONOO⁻) which further reacts with carbon dioxide (CO₂) to form nitrate (NO₃⁻), carbonate radical (CO₃⁻⁻) and nitrogen dioxide radical (•NO₂); or it (peroxynitrite) can react with thiol groups (Shiva, 2013). Nitric oxide also forms coordinate bonds with iron (Shiva, 2013).

Nitric oxide is produced by the enzyme nitric oxide synthase (NOS). The three isoforms of NOS include neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2) (Ziolo et al., 2008). NOS is a homodimer consisting of two identical subunits. NOS produces nitric oxide by converting L-arginine and molecular oxygen (O₂) to L-citrulline and nitric oxide. This reaction requires tetrahydrobiopterin (BH₄) as a cofactor of which is converted to (BH₂) in the process (Ziolo et al., 2008).
Considering its reactivity, defining the role of nitric oxide in the cardiovascular, immune and nervous systems have been active areas of research (Lancaster, 2020). Nitric oxide has been shown to play a particularly important role in the cardiovascular system by mediating vasorelaxation and cardioprotection.

### 3.2 Nitric Oxide Production in the Myocardium

In the heart, eNOS and nNOS are constitutively expressed and is regulated by Ca-calmodulin, whereas iNOS expression is induced during pathophysiological conditions (Ziolo et al., 2008). The NOS isoforms have been shown to have different effects on myocardial contraction. Specifically, nNOS is localized in the sarcoplasmic reticulum and acts on excitation-contraction coupling proteins, including L-type Ca\(^{2+}\), phospholamban and ryanodine receptor, to modulate contraction (Ziolo et al., 2008). Whereas eNOS is present within the caveolae and affects K\(^{+}\) current, Ca\(^{2+}\) current and troponin to modulate contraction. Lastly, iNOS has been identified in the cytosol during immune responses and is associated with contractile dysfunction (Ziolo et al., 2008).
3.3 Nitric Oxide and c-GMP dependent Cardioprotection

Nitric oxide has been shown to modulate cardioprotection by mediating levels of cyclic guanosine monophosphate (cGMP), and this discovery was awarded the Nobel Prize in 1998. While researching the role of the endothelium in bradykinin mediated vasorelaxation, the term for the undescribed molecule was coined “endothelium-derived relaxing factor” (EDRF) (Cherry, 1982). Shortly after, Furchgott predicted that EDRF functions through the GMP cycle. This was confirmed after review of previous studies associating nitrovasodilators with increased cyclic GMP (cGMP) levels and others showing that increased cyclic GMP levels causes smooth muscle relaxation by acetylcholine (Furchgott, 1996; Rapoport, 1983). Later research into some inhibitors of EDRF, including hemoglobin and methylene blue, as well as EDRF’s response to superoxide (O$_2^-$) led Furchgott to discover that this molecule was indeed nitric oxide (Furchgott, 1996). In summary, this research described how the nitric oxide produced in the endothelial cell enters the smooth muscle cell and activates soluble guanylyl cyclase and, in turn, increases cGMP levels (Furchgott, 1996).
Continued research into cGMP-dependent effects of nitric oxide has provided a mechanistic understanding of its role in protecting against pathological hypertrophy and reperfusion injury. Specifically, the increase in cGMP stimulates cGMP-dependent protein kinase type 1 (PKG 1), of which then inhibits the L-type calcium channels and, in turn, lessens the amount of calcium ions entering the cell. This suppresses the calcium dependent NFAT pathway which then reduces downstream hypertrophic gene expression (Kempf & Wollert, 2004; Schroder, 2003; Wollert, 2002). Additionally, research has shown that cGMP activity is important in protecting against reperfusion injury by controlling calcium ions levels during reperfusion, intracellular acidity, and mitochondrial permeability transition (Inserte & Garcia-Dorado, 2015).

3.4 Nitric Oxide and c-GMP independent Cardioprotection

Furthermore, nitric oxide also modulates cardioprotection by way of S-nitrosylation which is a post translational modification to the side chain of cysteine amino acids. Specifically, nitric oxide reacts with the thiol group to produce a S-nitrosothiol group (Evangelista et al., 2013). This reaction is reversible and multiple methods of denitrosylation have been described (Evangelista et al., 2013). This affects protein function by protecting the thiol group from oxidation as well as modifying the function of the overall protein. Evangelista et al. mentioned the effects as being “largely contextual” (Evangelista et al., 2013).
3.5. Nitric Oxide and Endothelial Dysfunction

Nitric oxide is an endothelial factor with known vasodilator functions. It has been well described that nitric oxide is a regulator of blood pressure, and disruptions to nitric oxide production are associated with hypertension (da Cunha Martins et al., 2018). Under hypertensive conditions, the body produces ROS, and it is believed that nitric oxide functions as an antioxidant. The oxidation of nitric oxide reduces the bioavailable nitric oxide needed to induce vasodilation and counteract increased blood pressure (Hermann, 2006; Luscher, 1986; Panza, 1990). Studies show that deletion of the gene encoding endothelial nitric oxide synthase (eNOS) results in systemic hypertension (Huang, 1995; Shesely, 1996). As such, direct impact on eNOS activity can also lead to endothelial dysfunction and hypertension.

3.6. Nitric Oxide and Cardiac Fibrosis

Inhibition of eNOS is associated with enhanced myocardial fibrosis through the recruitment and mobilization of fibroblasts (Kazakov et al., 2013). This is believed to be mediated by an increase in collagen and chemokine signaling (Kazakov et al., 2013).

4. Metallothionein

Metallothionein is a conserved protein that aids in the transport of essential metals like zinc and copper, redox control, and detoxification of heavy metals (Irvine et al.,
The protein is composed of many cysteine groups, and the thiols groups of these amino acids facilitate metal binding. In addition to containing many reduced cysteines groups, sequences of amino acids define two domains of the metallothionein protein, specifically the α cluster and the β cluster (Sabolic et al., 2010). It is at these clusters where metal binding occurs. Although the exact mechanisms of how the metal binding to metallothionein in humans remains unclear, Irvine et al. propose two possibilities; the clustered pathway and the beaded pathway (Irvine et al., 2016). The beaded pathway is associated with a cooperative mechanism where the clustered pathway is associated with noncooperative binding, and this differentiation is consistent with the observation that metallothionein interacts differently with essential and non-essential metals (Irvine et al., 2016).

There are four isoforms of metallothionein known to be expressed in humans; MT1, MT2, MT3, and MT4, and but MT1 and MT2 are the most commonly expressed isoforms across mammals (Sabolic et al., 2010).

Metallothionein expression is induced by several activating ligands including metals, glucocorticoids, inflammatory modulators, and free radicals that bind to different response elements (Sabolic et al. 2010). Most notably, the metalloregulatory transcription factor (MTF-1) binds to the metals response element (MRE) to drive transcription of the metallothionein gene (Sabolic et al., 2010). Specifically, Sabolic et al. describes a mechanism where zinc ions bind to apo-MTF-1, inducing phosphorylation of the MTF-1 protein which allows it to bind to the MRE region (Sabolic et al., 2010).
Interestingly, early studies and preliminary data suggest metallothionein concentration is higher in female organs. This indicates that there may be a hormonal factor at play (Sabolic et al., 2010).

4.1 Metallothionein and Redox Control

In addition to its role in activating metallothionein’s transcription factor, zinc also plays an important role when bound to the metallothionein protein. Metallothionein is a potent acceptor of zinc intracellularly, and the two bind to form a stable ZnMT complex (Sabolic et al., 2010). This complex helps to maintain the redox state of the cell. (Sabolic et al., 2010) During oxidative stress, zinc is released from the complex, and the free zinc binds to apo-MTF-1 to drive increased expression of the metallothionein gene and, in turn, the metallothionein protein functions as an antioxidant by scavenging free radicals and releasing bound glutathione (Sabolic et al., 2010).

4.2. Metallothionein and Ischemia-Reperfusion Injury

Metallothionein has been shown to play an important role in protection against ischemic injury in the heart. A study using the Langendorff perfusion model compared ischemia-reperfusion injury between metallothionein overexpressed mice and nontransgenic controls. The metallothionein overexpression mice had less infarcted tissue, lower creatine kinase levels and improved contractile force postischemia than controls (Kang, 1999). Researchers attributed metallothionein’s protection to its ability to
combat oxidative stress as a potent free radical scavenger (Kang, 1999). In a subsequent study, Kang et al. used an open-chest coronary artery occlusion and reperfusion model to study apoptosis in metallothionein overexpression mice and nontransgenic controls (Kang, 2003). The metallothionein overexpressed mice had less infarcted tissue, reduced lipid peroxidation and inhibited cytochrome c release and caspase-3 activation (Kang, 2003). Overall, these results as well as those of other studies point to a protective role for metallothionein against ischemia reperfusion damage, likely through its ability to scavenge free radicals and reduce oxidative stress.

5. Cardiovascular Disease Risk Factors

Research into CVD risk factors became a priority in the middle part of the twentieth century (Mahmood et al., 2014). In 1948, researchers started the Framingham Heart study in attempt to better understand the risk factors of CVD (Mahmood et al., 2014). They created a multivariable logistic model to calculate a risk score with an aim to quantify an individual’s risk of developing cardiovascular disease after ten years. In the early version, the input variables included total cholesterol, electrocardiogram abnormalities, age, weight, haemoglobin, systolic blood pressure and cigarette smoking (Mahmood et al., 2014). This demonstrates a clear focus on the modifiable risk factors of CVD, particularly those attributed to an individual’s lifestyle choices. Likewise, today the American College of Cardiology and the American Heart Association routinely highlight guidelines on how to manage these modifiable risk factors of CVD, such as maintaining a balanced diet, routine physical activity and preventing tobacco use (Arnet et al. 2019).
5.1. Environmental Cardiovascular Disease Risk Factors

Even with effective maintenance of these risk factors for CVD, prevalence remains high. This suggests that other factors are in play beyond those that are modifiable, of which we include environmental factors (Cosselman et al., 2015). Defining the role of environmental factors in disease is a primary focus of environmental health research. Specially, the concept of environmentally related CVD is based on prior research that associates CVD with exposure to toxic substances in the environment such as particulate matter, metals and pollutant gases.

The heart’s high energy demand and electrophysiological function may cause it to be particularly sensitive to these toxic substances. Cardiotoxicity generally results in decreased cardiac output and blood flow in peripheral tissue by targeting the biochemistry, energy metabolism, cellular structure and function, electrophysiology, and contractility of the heart (Mahmood et al., 2014). This range of potential targets and the possibility of interaction underscores the need for additional research into the ever-increasing number of environmental contaminants.

5.2. Metal Exposure and Cardiovascular Disease

Evidence suggests that exposure to certain metals is strongly associated with CVD. Research has identified toxicity by essential metals at disproportionate levels and nonessential metals at a range of doses and from different species of these metals and
metalloids. Particularly relevant CVD actors include mercury, cadmium, lead and arsenic, all of which have been associated with alterations to the structure and function of the heart (Wang et al., 2019). Given the ubiquitous nature of these metals in our environment and increasing presence across industries and manufacturing processes, research into their associations with CVD is critical while attempting to define all risk factors for CVD.

6. Cadmium

Cadmium is a transition metal naturally found in the earth’s crust and is a divalent cation in its free form (Morrow H. 2000). Although it is a natural substance, cadmium poses a threat to human health and is listed seventh on the ASTDR’s Substance Priority List (ATSDR, 2020). It is mobilized into our air, water and food as a byproduct of mining, refining, fertilizer application, car exhaust, waste management and is used as an additive in several consumer products (Faroon et al., 2012). Specifically, cadmium exists as particles or vapors in air, as organic compounds in soil, and as hydrated ions or ionic complexes in water (Faroon et al., 2012). Cadmium is known to mobilize and speciate through the environment, allowing it to ultimately accumulate in crops and sources of drinking water (Faroon et al., 2012).

6.1. Human Exposure to Cadmium
Human exposure parameters to cadmium are dependent on many factors including an individual’s geographical location, occupation, diet, smoking status, etc. Cigarette smoke and dietary intake are the two major sources of cadmium exposure (Faroon et al., 2012). Intake and uptake value of cadmium differ, because not all cadmium that enters our body via food and water is absorbed. According to an International Programme on Chemical Safety (IPCS) report, the daily intake of cadmium for uncontaminated areas is > 1 μg/day from water and 10-25 μg from food (Fridberg et al. 1992). Whereas, and according to this same report, the total daily intake of cadmium for contaminated areas can reach as high as 150-200 μg/day (Fridberg et al. 1992). Given prior research highlighting the ambiguity surrounding the cardiotoxic effects of cadmium after inhalation exposure, the following study focuses on oral exposure (Tellez-Plaza, Jones, et al., 2013).

6.2. Absorption of Cadmium

Analogous to essential metals, the majority of ingested cadmium is absorbed into the body via the upper intestine (Bressler et al., 2004). Prior studies provide strong evidence that toxic metals are transported into the body via the same transport mechanisms of iron (Bressler et al., 2004; Flanagan et al., 1980) A review by Bressler et al. present research that suggest bound cadmium separates from proteins like metallothionein and phytochelatin in the acidic gastric juice, and this free cadmium ion is transported into intestinal epithelium via divalent metal transporter 1 (DMT1), the same mechanism involved in the transport of iron (Bannon et al., 2003; Bressler et al., 2004;
Gunshin et al., 1997; Klein et al., 1988). Once absorbed at the luminal surface of the cell, the free cadmium may then be trafficked to the basal surface by calbindin and then transported across the basal surface potentially by Ireg1 or Ca-ATPase (Bressler et al., 2004).

Once transported in the blood to the heart, cadmium is believed to be absorbed into the cardiomyocytes via Ca$^{2+}$, Cu and Zn transport processes, including membrane sulfhydryl groups and calcium transporter channels (Limaye & Shaikh, 1999).

### 6.3. Cadmium Accumulation in Human Tissues

As with other contaminants, biomarkers including an individual’s nails, hair, blood and urine can be used to detect cadmium exposure (Faroon et al., 2012). Specifically, cadmium can be detected in blood to measure recent exposure, and it can be detected in urine to measure recent and prior exposures (Faroon et al., 2012). The difference in cadmium levels between the blood and urine demonstrate that cadmium is accumulating in tissue, and animal studies have confirmed cadmium accumulation showing it at detectable levels in the heart, kidney and liver tissue (Young et al., 2019). Additionally, a study into cadmium exposure and aortic dilation observed higher concentrations of cadmium accumulation in the medial layer of the aortic lining from individuals exposed to cadmium via smoke inhalation (Abu-Hayyeh et al., 2001).

### 6.4. Epidemiological Studies into Cadmium Toxicity and Cardiovascular Disease
An abundance of epidemiological studies present cadmium as a potential driver of the growing risk and incidence of CVD. Although the following is not a comprehensive review of these studies, it serves to represent the current evidence of this association. Many studies suggest a positive association between cadmium exposure and hypertension, including a population-based study that associated urinary cadmium levels with hypertension (Swaddiwudhipong et al., 2010). Also, case-control studies associated cadmium exposure and urinary cadmium levels with atherosclerosis (Houtman, 1993; Navas-Acien et al., 2005). Considering atherosclerosis is a risk factor for hypertension, this reported association between cadmium exposure and atherosclerosis may suggest a potential mechanism to be explored in future studies. Additionally, epidemiological studies associate cadmium exposure with incidence of cardiovascular diseases such as coronary artery disease, myocardial infarction, and heart failure. A prospective cohort study associated cadmium exposure with increased mortality as well as increase cases of incident conditions (Tellez-Plaza, Guallar, et al., 2013). Another study that analyzed data from the 1999-2006 National Health and Nutrition Examination Survey showed an association between cadmium levels in the blood and increased prevalence of heart failure and stroke (Peters et al., 2010). Across-sectional analysis of the data from the 1988-1994 National Health and Nutrition Examination Survey showed an association between urinary cadmium levels and increased coronary heart disease and myocardial infarction. Interestingly this study also suggested the presence of a sex difference, by highlighting that women with urinary cadmium levels had greater odds of having had myocardial infarction than males (Everett & Frithsen, 2008). Furthermore, a prospective
population study showed an association between cadmium and systolic lead exposure with left ventricle dysfunction (Yang et al., 2017).

6.5. Model for Understanding Molecular Cardiotoxicity

In a review introducing the molecular and cellular mechanisms of cardiotoxicity, Kang provides a model overviewing how an environmental toxic insult progresses to the development of hypertrophy and ultimately heart failure (Kang, 2001). This framework suggests that toxic substances from our environment drive biochemical alterations, notably via a disruption to homeostatic ion levels (Kang, 2001). This ion imbalance can cause electrophysiological changes, cell death and an increased activity of transcription factors that induce expression of hypertrophic genes (Kang, 2001). As depicted, hypertrophy then causes tissue hypoperfusion and, in turn, the activation of compensatory mechanisms (Kang, 2001). These compensatory mechanism breakdown and counter regulatory responses as well as an influx of cytokines can induce myocardial remodeling and further cell death (Kang, 2001). This ultimately drives heart failure (Kang, 2001).
6.6 Cadmium Exposure and Cardiomyocyte Death

A recent study into the effects of intragastric administration of 5 mg kg/bw/day of CdCl₂ on myocardial tissue and erythrocytes of albino Wistar rats showed that cadmium exposure induces myocyte apoptosis. Results from qPCR analysis showed a significant increase in expression of Bax and Caspase 3 mRNA and a decrease in Bcl2 mRNA, and further experiments showed consistent changes in Bcl2, Bax and caspase protein expression (Ghosh & N, 2018). Additionally, this study showed increased DNA fragmentation and increased cleaved caspase 3, providing further evidence that cadmium exposure induces cell death via apoptosis (Ghosh & N, 2018). Another study into the
effects of cadmium and high cholesterol levels exposure also reported cell death. Results from an in vivo exposure demonstrated that cadmium exposure induced DNA strand breaks, and further in vitro work confirmed the presence of mitochondrial depolarization (Turkcan et al., 2015). Turkcan et al. suggested that their exposure induced cell death primarily via necrosis (Turkcan et al., 2015). Although the exposure differed across these two studies, further work is needed to determine whether and when cadmium is inducing cell death via apoptosis or programmed necrosis and what factors influence this distinction.

6.7 Cadmium Exposure and Oxidant-Antioxidant Imbalance

Wistar rats exposed to intragastric administration of 5 mg kg/bw/ day of CdCl₂ also showed reduced levels and activity of antioxidants in cardiac tissue, plasma, hemolysate and erythrocyte membrane (Ghosh & N, 2018). Results showed reduced levels of α-tocopherol, ascorbic acid and glutathione as well as reduced activity of superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase (Ghosh & N, 2018). Another study into the effect of a diet supplemented with 140 mg/kg CdCl₂ on the chicken heart showed that cadmium exposure results in oxidative stress and heart damage (Yu et al., 2021). Results from different biochemical assays of the same study, RNA sequencing and real time quantitative PCR showed signs of decreased glutathione, peroxidase and superoxide dismutase; an increase in malonaldehyde; as well as other measures confirming the presence of oxidative stress (Yu et al., 2021).
6.8 Cadmium Exposure and Cardiomyocyte Contractility

Live cell imaging was used to measure for changes in cardiomyocyte contractility after HL-1 cells (a cardiac muscle cell line) were exposed to 2.5 μmol/l and 5 μmol/l CdCl₂ (Turkcan et al., 2015). Results from video analysis showed that HL-1 cells exposed to cadmium for 48 hours had decreased and slower beating, suggesting cadmium reduces the contractility of the cardiomyocyte (Turkcan et al., 2015).

6.9 Cadmium Exposure and Hypertension

As mentioned above, epidemiological studies have associated cadmium exposure to hypertension in humans (Eum et al., 2008; Franceschini et al., 2017; "<Mortality in the United States, 2017.pdf>", ; Swaddiwudhipong et al., 2010) (Perry et al. 1955). However, this association has not been observed in all studies. A recent systematic review on the association between cadmium exposure, blood pressure and hypertension highlighted the conflicting results and the need for more mechanistic studies (Martins et al., 2021). In comparison to the epidemiological evidence of this association, animal and mechanistic studies measuring blood pressure are limited.

6.10 Cadmium Exposure and Disruption of Calcium Activity

Prior research suggests that cadmium interferes with calcium cycling within the heart. A study into the effects of perfusing male Sprague-Dawley rats with 0.03 μM or 3
μM CdCl₂ and various calcium concentrations showed that cadmium induces toxicity by altering contractility, energy metabolism and excitability in a calcium dependent manner (Prentice et al., 1984). In terms of the observed contractile and electrical effects, hearts exposed to 3 μM CdCl₂ showed reduced maximal contractile tension, lower rate of tension development, longer delay in conduction through the AV node, and longer activation time of the ventricular mass (Prentice et al., 1984). In terms of the effects on energy metabolism, hearts exposed to 3 μM CdCl₂ showed reduced levels of high energy phosphates and increased levels of low energy phosphates and increased accumulation of glycolytic intermediates as well as a lower coronary flow rate (Prentice et al., 1984). This study also discovered that these effects occur in an inverse relationship to the calcium concentration in the perfusate, which suggests a relationship between cadmium toxicity and extracellular calcium concentration (Prentice et al., 1984).

6.11. Cadmium Exposure and Cardiac Remodeling

A study showed that coexposure to a high dose of cadmium and high-fat diet (HFD) induced cardiac fibrosis and hypertrophy in female mice independent of oxidative stress (Liang et al., 2019). Sections of hearts stained with fluorescein-conjugated wheat germ agglutinin revealed hypertrophy in female hearts exposed to cadmium and HFD but not in hearts exposed to each of these variables individually (Liang et al., 2019). The presence of hypertrophy was confirmed with increased levels of atrial natriuretic peptide (ANP) protein and β-myosin heavy chain (β-MHC) levels (Liang et al., 2019). Sections stained with Sirius red revealed fibrosis in females exposed to cadmium and HFD, and
collagen deposition was confirmed with an increase in collagen alpha protein mRNA levels (Liang et al., 2019).

7. Public Health Significance Statement

The high prevalence of cardiovascular disease demands advancements in medical treatment as well as promotion and support of public health efforts aimed at prevention of these diseases. In order to be most productive, efforts seeking to better characterize already known risk factors as well as identify new ones are critical in developing the most comprehensive prevention practices. Given the significance of environmental influences on cardiovascular health, further research is needed to identify and define the effects of these agents. In particular, this study seeks to characterize the cardiotoxic effects of cadmium in the interest of not only aiding in the conditional adjustment to clinical assessment but also in the risk management and advocacy efforts intended to limit exposure and protect health.
CHAPTER 2

In the present study, we sought to further our current understanding of the cardiotoxic effects of cadmium. There are clear knowledge gaps in the literature, and the present study was novel in that it studied into the chronic effects of cadmium after an 8-week exposure to a relevant dose of cadmium in C57BL/6J mice. Accordingly, we leveraged our skills, expertise and lab facilities to test our overall hypothesis that exposure to cadmium will (1) provoke a fibrotic and hypertrophic phenotype and (2) exacerbate I/R injury in both sexes. Considering the known sex differences in cardiac physiology, we ran two parallel studies with one measuring for impairments to the cardiovascular system in females and the other for that in males.

1. Research Design and Methodology

1.1 Housing Conditions and Cadmium Chloride Exposure

This study consisted of three cohorts of 20 mice with 5 female control, 5 male control, 5 female cadmium and 5 male cadmium per cohort. All C57BL/6J mice were acquired from Jackson Laboratories and were seven weeks old upon arrival. The mice were housed together in their respective groups in facilities maintained by the Research Animal Resources of Johns Hopkins University. All mice were provided ad libitum with AIN-93G chow acquired from Research Diet Inc. and Nestle Pure Life water provided to each cage in newly purchased 100-mL glass bottles. Following a 1-week pre-exposure period, 10 of the eight-week-old mice were given drinking water containing 5,000 ppb of...
cadmium chloride (CdCl₂) purchased from Aldrich Chemistry. The chow in each cage was replenished to a mass of 75.0 grams three times a week. The drinking water was replaced and filled to 75.0 mL three times a week to minimize the potential for oxidation.

1.2 Animal Sacrifice

The described anesthesia and excision protocols were followed as routinely performed in the Kohr lab and in a manner compliant with the ethical standards enforced by Johns Hopkins University Animal Care and Use Committee. Following the eight-week exposure period, mice were anesthetized with a mixture of 0.1 mL ketamine, 0.5 mL xylazine, and 0.1 mL sodium chloride and anticoagulated with injection of 0.1 mL heparin into the superior vena cava. Mice were confirmed to be appropriately by testing for the loss of the pedal reflex. Upon excision of the heart, blood was drawn from the superior vena cava and that of which pooled within the thoracic cavity, additional organs were excised (kidneys, front liver lobe, lungs, brain and fat) and the tibia bone was isolated from the right leg of each mouse.

1.3 Langendorff Preparation and Krebs-Henseleit Buffer

Upon excision, hearts were temporarily placed in cold perfusate buffer until it was cannulated on the Langendorff apparatus via the aorta. The hearts were cannulated 1-2 mm above the aortic valve to ensure the needle did not break through the aortic valve and enter the left atria (Botker et al., 2018). Once cannulated and upon opening of the
perfusate buffer flow valve, the flowing buffer forces the three leaflets of the aortic valve to close, shunting the perfusate buffer into the coronary arteries, and in turn supplying the heart with the nutrients and oxygen necessary for sustained function ex vivo (Botker et al., 2018).

The 1X Krebs-Henseleit buffer is prepared by adding a salt stock mixture (14.0 g sodium chloride, 4.2 g sodium bicarbonate and 4.0 g of glucose), 20 mL of 100X Krebs buffer (35.04 g potassium chloride, 16.33 g potassium dihydrogen phosphate and hepta-hydrate magnesium sulfate dissolved in 500 mL of MilliQ water) and 1,750 μL calcium chloride dissolved in 2 L of MilliQ water. This solution is mixed for 5 minutes and filtered through a membrane using a filtration apparatus. Our Langendorff adopts the constant perfusion pressure model by maintaining a perfusate pressure of 100 cm of H2O (Botker et al., 2018). Additionally, the perfusate buffer was pre-gassed and bubbled with 95%O₂/5%CO₂, maintained at a pH of 7.4 and at a temperature of 37°C. 27 (Botker et al., 2018).

Upon successful cannulation, hearts deemed for molecular analysis were perfused for five minutes, weighed and stored in -80°C, and the others were subjected to the full I/R protocol as described below.

1.4 Ischemia-Reperfusion Protocol on Langendorff

The ischemia-reperfusion (I/R) protocol applied to this study consists of a 20-minute period of perfusion, a 20-minute period of ischemia and a 40-minute or 90-minute period of reperfusion. Hearts were exposed to ischemia ex vivo by shutting the perfusate valve on the Langendorff, which halts the delivery of oxygen and nutrients to the heart.
In order to measure contractile function, the left atria appendage was removed and a small polyethylene balloon filled with water was inserted through the left atria and mitral valve and positioned within the left ventricle of the heart. The pressure exerted onto the balloon is converted from mechanical energy to electrical energy by a pressure transducer and presented as left ventricular developed pressure (LVDP), heart rate (HR) and rate pressure product (RPP) in LabChart software purchased by ADInstruments. (Botker et al., 2018) The pre-ischemic and reperfusion RPP was used to calculate functional recovery, which is represented as a percentage of cardiac function that is restored after ischemia and during reperfusion.

**Rate Pressure Product:** $RPP = LVDP \times HR$

**Functional Recovery** $= \frac{\text{Reperfusion RPP} \times \text{Perfusion (Baseline) RPP}}{100}$

After the I/R protocol, the hearts were perfused with 2,3,5-triphenyl tetrazolium chloride (TTC) solution (0.1 g 10 mL 1X Krebs-Henseleit buffer) and left to incubate at 37°C for 20 minutes. The dehydrogenases in live tissue convert the TTC to a red product, whereas the infarcted tissue remains white. Then, the hearts were weighed and stored in 1.0 mL of formalin in cryovial at room temperature, and later blinded and sliced into 1.0 mm coronal sections using an acrylic mouse heart slicer matrix acquired from Zivic Instruments. The top and bottom of each slice from every heart was imaged using a Leica Stereoscope, processed in Adobe Photoshop and analyzed using Image J software downloaded from the National Institute of Health. Ultimately, the data provided by the
image processor was used to calculate the percent of infarcted tissue on each slice of the heart and compared between the control and cadmium exposed hearts of both slices.

1.5 Blood Pressure Analysis

The BP-2000 Blood Pressure Analysis System is a tail-cuff pressure transduction apparatus purchased from Visitech System (Visitech Systems, 2021, How It Works section). Similar to a sphygmanomanometer used in a clinical setting to measure blood pressure within the brachial artery, this system is a non-invasive method of measuring blood pressure within the tail artery of a mouse (Visitech Systems, 2021, How It Works section). The Visitech website provides a detailed review of this technology (Visitech Systems, 2021, How It Works section). Specifically, the BP-2000 leverages photoplethysmography technology (Visitech Systems, 2021, How It Works section). This is a method where light is transmitted from an emitter, passed through the tail and measured at a receiver (Visitech Systems, 2021, How It Works section). A heart contraction produces a pressure wave that travels throughout the vasculature and causes slight dilation in passing (Visitech Systems, 2021, How It Works section). This slight dilation of the vessel causes the emitted light to scatter. The tail cuff is inflated to measure diastolic and systolic blood pressure (Visitech Systems, 2021, How It Works section). The pressure at which the waveform begins to decrease is the diastolic blood pressure (Visitech Systems, 2021, How It Works section). It is at this pressure where the inflated cuff has started to restrict the dilation of the blood vessel in response to the passing pressure wave, and, in turn, limits the scattering of light (Visitech Systems, 2021,
How It Works section). Furthermore, the pressure at which the waveform decreases to a constant level is the systolic blood pressure (Visitech Systems, 2021, How It Works section). It is at this pressure where the inflated cuff has completely restricted the dilation of the blood vessel in response to the passing pressure wave (Visitech Systems, 2021, How It Works section). In other words, the applied force from the pressure cuff is greater than the pressure exerted from the blood on the walls of the blood vessel.

To collect the measurements, mice were placed under a darkened animal restrainer and on top of a warm platform. The mouse tail was gently slid through the occlusion cuff and taped in place. Prior to collection of data recording, the mice were trained on the entire protocol to reduce any stress effect on the later blood pressure measurements. 20 -25 blood pressure measurements were taken for each mouse twice a week. After collection, each tracing was analyzed. Tracings that were labeled as being “outliers”, “excessive movement”, “pulse not detected” and “indistinct signal” were excluded from the data set. The systolic lines of the remaining tracings were adjusted as necessary. These measurements were averaged for the for each day and the average systolic pressure readings were reported per week.

1.6 Echocardiography

Mr. Nandan Wang of the Small Animal Cardiovascular Phenotyping and Model Core at Johns Hopkins Medicine performed echocardiography of the mouse hearts three times (0, 4, and 8 weeks) for two of our cohorts (1 and 3). A Vevo 2100 with a 40-MHz linear transducer was used to perform the echocardiogram. It captured a short-axis view
of the mid-papillary muscles of the left ventricle (LV) at a sweep speed of 200 mm/s.

This study collected measurements of interventricular septum thickness at end-diastole (IVS;d), interventricular septum thickness at end-systole (IVS;s), left ventricular internal dimension at end-diastole (LVID;d), left ventricular internal dimension at end-systole (LVID;s), left ventricular posterior wall thickness at end-diastole (LVPW;d), and left ventricular posterior wall thickness at end-systole (LVPW;s). These values were used to calculate corrected left ventricle mass (LV mass) and functional measures including ejection fraction (EF) and fractional shortening (FS).

Ejection Fraction = \(((LVID;d^2 - LVID;s^2) / LVID;d^2) \times 100\)

Fractional Shortening = \(((LVID;d - LVID;s)/LVID;d) \times 100\)

Left Ventricle Mass = \((\text{IVS;d} + \text{LVID;d} + \text{LVPW;d})^3 - (\text{LVID;d})^3\)

Corrected Left Ventricle Mass = Left Ventricle Mass \times 0.8

1.7 Tissue Homogenization and Bradford Assay

To prepare 10 mL of homogenization buffer, 9 mL Milli-Q, 1 mL 10X Cell Signaling lysis buffer, 100 µL protein inhibitor cocktail purchased by Thermo Fisher Scientific and 5 µL neocuporine were mixed into solution on ice in a 15 mL falcon tube. Tissue samples that were being stored at -80 °C were transferred into homogenization tubes containing 1 mL of the prepared homogenization buffer and then placed into the Precellys Evolution tissue homogenizer purchased from Bertin Instruments. Once the homogenizer chamber was sufficiently cooled with dry ice, the appropriate cycle was
selected and run. This homogenized tissue was then centrifuged at 15,000 rpm for 10 minutes and was left on ice for 15 minutes before aliquoting the supernatant. This supernatant was used for subsequent protein quantification and specific protein detection assays.

The Bradford assay was prepared by first creating four standards of the following solutions on ice in individual tubes containing the following quantities in the respective order: MilliQ-water (1.018 mL, 1.013 mL, 1.008 mL, and 1.000 mL), bovine serum albumin (0 μL, 5 μL, 10 μL, 20 μL), and 250 μL of acidified Coomassie brilliant blue G-250 dye. Then two tubes of solution containing the homogenate were prepared by mixing 1.018 mL Milli-Q water, 2 μL of tissue homogenate and 250 μL of acidified Coomassie brilliant blue 5-250 dye. All tubes were inverted and kept on ice for 5 minutes. Then a 96-well plate was prepared containing 200 μL of standard solution in three wells per standard and 200 μL of each sample solution into two wells per sample. The prepared 96-well plate was then transferred to the spectrophotometer and ran with absorption set to a wavelength of 600. The output was exported to an excel file and further analyzed to calculate protein quantities of the tissue samples.

1.8 Western Blotting

Kidney tissue samples were diluted in Milli-Q water and loading dye. This solution was placed into a heat block and set at 95°C for 5 minutes to denature the proteins. After heating, a specific volume of this sample, calculated using the formula provided below, was loaded into the wells of NuPage 4-12% Bis-Tris gel. Electrophoresis
followed at 75 V for 20 minutes and at 175 V for 1 hour 40 minutes. The proteins were then transferred from the gel to polyvinylidene difloride (PVDF) membrane with the current set to 220 mA for 1 hour 30 minutes. No-stain protein labeling reagent was applied to the membrane and imaged to visualize total protein on the membrane post-transfer. Membrane proteins were blocked in 5% milk at 4°C overnight and cut along 80 kDa to separate the segment of membrane with eNOS from the segment with protein kinase B (Akt). Each segment was incubated with their respective primary antibody (1:1000) diluted in 5% BSA at 4°C overnight and then incubated with secondary antibody (1:5000) diluted in 5% BSA at room temperature for 1 hour. The membrane was then incubated in Super Signal West Pico PLUS Chemiluminescent Substrate for 5 minutes and imaged on the iBright Imaging System. Densitometry was performed using Image J Software acquired from the National Institute of Health.

\[
\text{Protein Volume to Load into Gel} = \frac{30 \mu g}{\text{Protein Concentration}}
\]

1.9 Statistics

All statistical analysis was performed in GraphPad Prism 9 software.

For data with the variable of time, a two-way analysis of variance (ANOVA) was used and the p-value for the interaction term was reported. In terms of the echocardiography data: if that interaction term was significant, p-values for any significant differences measured by a Multiple Comparisons Tests between the control and cadmium group was also provided.
For all else, the data was first tested for normality with the Shapiro-Wilk test. If both the control group and cadmium exposed group passed the normality test, an unpaired t test was used to compare the means of the two group. A Mann-Whitney test was used if one of or both the control group and cadmium exposed group failed the normality test instead.

2. Results

2.1. CdCl₂ exposure does not alter body weight in C57BL/6J mice

We examined the effect of cadmium exposure on body weight in male and female C57BL/6J mice, but found no difference between control and cadmium treated mice for both sexes (Fig. 1).

![Figure 1](image_url)

Figure 1: (A-C) There was no significant difference in the change in body weight change in female C57BL/6J mice exposed to 5 ppm CdCl₂ (n=15) for 8 weeks compared to the
control mice (n=15). (D-F) Similarly, there was no significant difference in the change in body weight change in male C57BL/6J mice exposed to 5 ppm CdCl₂ (n=15) for 8 weeks compared to the control mice (n=15) to report.

2.2. CdCl₂ exposure does not alter food or water consumption in C57BL/6J mice

We examined the effect of cadmium exposure on food and water consumption in male and female C57BL/6J mice, but found no difference between control and cadmium treated mice for both sexes (Fig. 2&3).

Figure 2: (A-F) There was no significant difference in the change in food consumption in female and male C57BL/6J mice exposed to 5 ppm CdCl₂ (n=15/sex) for 8 weeks compared to the control mice (n=15/sex). The slight difference observed in cohort 1 was deemed as negligible, because the difference was not observed in cohorts 2 and 3.
Figure 3: (A-F) There was no significant difference in the change in water consumption in female and male C57BL/6J mice exposed to 5 ppm CdCl₂ (n=15/sex) for 8 weeks compared to the control mice (n=15/sex). As mentioned in regards to food consumption, the slight difference observed in cohort 1 was deemed as negligible, because the difference was not observed in cohorts 2 and 3.

2.3. CdCl₂ exposure does not alter systolic blood pressure in C57BL/6J mice

We next examined the effect of cadmium exposure on blood pressure in male and female C57BL/6J mice, but found no difference between control and cadmium treated mice for both sexes (Fig. 4). Measuring for changes in blood pressure is important, because a change in blood pressure may signify a pathology that alters cardiac output or peripheral resistance.
Figure 4: (A-B) There was no physiologically or statistically significant difference in the change in systolic blood pressure (mmHg) in female and male C57BL/6J mice exposed to 5 ppm CdCl₂ (n=5/sex) for 8 weeks compared to the control mice (n=5/sex). (p values = 0.8466 and 0.3176 respectively)
We used echocardiography to measure for alterations in the following cardiac dimensions: interventricular septum thickness at end-diastole, interventricular septum thickness at end-systole, left ventricular internal dimension at end-diastole, left ventricular internal dimension at end-systole, left ventricular posterior wall thickness at end diastole and, left ventricular posterior wall thickness at end systole. We used these values to calculate ejection fraction and fractional shortening, which provides insight into how cadmium disrupts cardiac contractility. Echocardiography was used to measure these parameters in vivo and at three different time points. Most notably, cadmium
exposure reduces interventricular septum thickness, increased left ventricle internal dimension and reduced ejection fraction and fractional shortening in male C57BL/6J mice. (Fig. 6). These effects were absent in female mice.

Figure 6: A) There was no statistically significant change in IVS;d over time in the female and male groups exposed to 5 ppm CdCl₂ (n=10) compared to the control mice (n=10); (p values = 0.7794 and 0.0626 respectively.) [ANOVA test]

Figure 6: B) There was no statistically significant change in IVS;s over time in female mice exposed to 5 ppm CdCl₂ (n=10); (p value = 0.4426). [ANOVA test] However, there was a statistically significant change in IVS;s over time in the male group exposed to 5 ppm CdCl₂ (n=10); (p value = 0.0117). [ANOVA test] Comparing IVS;s of the male group at 0 weeks versus 8 weeks showed a statistically significant difference in the
control group (*p value = 0.0167) but not in the group exposed to 5 ppm CdCl₂ (n=10). (p value = 0.0869). [Multiple comparisons test]

C

**Left ventricular internal dimension at end-diastole**

Figure 6: C) There was a statistically significant change in LVID;d over time in the female group exposed to 5 ppm CdCl₂ (n=10). (*p value = 0.0296) [ANOVA test]
Comparing LVID;d of the female group at 0 weeks versus 4 weeks showed a statistically significant difference in the control group (n=10) (*p value = 0.0063) but not in the female group exposed to 5 ppm CdCl₂ (n=10). (p value = 0.9526) [Multiple comparisons test] However, there was no statistically significant change in LVID;d over time in the male group exposed to 5 ppm CdCl₂ (n=10). (p value = 0.7610) [ANOVA test]

D

**Left ventricular internal dimension at end-systole**

Figure 6: D) There was no statistically significant change in LVID;s over time in female and male mice exposed to 5 ppm CdCl₂ (n=10) compared to the control mice (n=10); (p
values = 0.0532 and 0.0524 respectively.) [ANOVA test] Nonetheless and given the p values are so close to reaching significance, further statistical analysis was still performed. Comparing LVID;s of the female group at 0 weeks versus 8 week showed a statistically significant difference in the control group (*p-value 0.0001) but not in the female group exposed to 5 ppm CdCl₂ (n=10) (p value = 0.1263). [Multiple comparisons test] Additionally, comparing LVID;s of the male group at 0 weeks versus 4 weeks and 8 weeks showed a statistically significant difference in the male group exposed to 5 ppm CdCl₂ (n=10) ( *p values = 0.0412 and 0.0360 respectively) but not in the male control group (n=10) (p values = 0.1821 and 0.0601 respectively). [Multiple comparisons test]

**Figure 6:** E) There was no statistically significant change in LVPW;d over time in the female and male groups exposed to 5 ppm CdCl₂ (n=10) compared to the control groups (n=10); (p values = 0.7794 and 0.0626 respectively.) [ANOVA test]
Figure 6: F) There was no statistically significant change in LVPWs over time in the female and male groups exposed to 5 ppm CdCl₂ (n=10) compared to the control groups (n=10); (p values = 0.7909 and 0.9849 respectively.) [Multiple comparisons test]

Figure 6: G) There was no statistically significant change in EF over time in the female group exposed to 5 ppm CdCl₂ (n=10) compared to the control group (n=10); (p value = 0.1421). [ANOVA test] However, there is a statistically significant change in EF over time in the male group exposed to 5 ppm CdCl₂ (n=10) compared to the control group (n=10); (*p value = 0.0056). [ANOVA test] Comparing EF of the male group at 0 weeks versus 4 weeks showed a statistically significant difference in the male control group (p value = 0.0080) but not in the male group exposed to 5 ppm CdCl₂ (n=10) (n=10); (p value = 0.1712). [Multiple comparisons test]
Figure 6: H) There was no statistically significant change in FS over time in the female group exposed to 5 ppm CdCl₂ (n=10) compared to the control group (n=10); (p value = 0.1615). [ANOVA test] However, there is a statistically significant change in FS over time in the male group exposed to 5 ppm CdCl₂ (n=10) compared to the control group (n=10); (*p value = 0.0036). [ANOVA test] Comparing FS of the male group at 0 weeks versus 4 weeks showed a statistically significant difference in the male control group (p value = 0.0087) but not in the male group exposed to 5 ppm CdCl₂ (n=10) (n=10); (p value = 0.2383). [Multiple comparisons test]

Figure 6: I) There was no statistically significant change in LVmass over time in the female and male groups exposed to 5 ppm CdCl₂ (n=10) compared to the control groups (n=10); (p values = 0.5872 and 0.4347 respectively.) [ANOVA test]
2.5. *Cd* exposure does not result in cardiac hypertrophy in C57BL/6J mice

Since we noted some changes in cardiac structure and function, we next measured for changes in the heart weight-to-tibia length ratio as a measure of cardiac hypertrophy. However, no changes in the heart weight-to-tibia length ratio were noted in either male or female mice.

![Graph showing heart weight-to-tibia length ratio](image)

Figure 7: (A-B) There was no statistically significant change in heart weight-to-tibia length over time in the female and male groups exposed to 5 ppm CdCl₂ (n=15/sex) compared to the control groups (n=15/sex); (p values = 0.4520 and > 0.9999 respectively). [Unpaired t test; Mann-Whitney test]

2.6. *CdCl₂* exposure causes a slight, but not statistically significant, decrease in infarct size in males after ischemia reperfusion injury in C57BL/6J mice
Since cadmium has been associated with alterations to ischemic susceptibility in the heart, we used an ex vivo model of ischemia-reperfusion injury and assessed changes in infarct size as a measure of cell death. Such a difference in infarct size would suggest that cadmium may worsen ischemia-reperfusion injury or effect processes that protect against this damage. However, cadmium exposure did not alter the response to ischemia-reperfusion injury in either sex with statistical significance.

Figure 8: (A-B) There was no statistically significant difference in infarct size in the female group exposed to 5 ppm CdCl₂ (n=10) compared to the control groups (n=7); (p value = 0.8868). [Mann-Whitney test] Similarly, there was no statistically significant difference in infarct size in the male group exposed to 5 ppm CdCl₂ (n=9) compared to the control group (n=6); (p value = 0.1797). [Unpaired t test]

2.7. \textit{CdCl₂} exposure does not induce a change in eNOS and Akt expression in female and male kidneys of \textit{C57BL/6J} mice
We measured for changes in eNOS expression in the kidney because it is known to modulate cardioprotection through the production of nitric oxide. Likewise, we measured for changes in Akt expression because Akt is known to activate eNOS. Changes in renal expression of these proteins may indicate a similar change in the heart that result confers or abrogates cardioprotection against ischemia-reperfusion injury in the heart. However, we did not detect any differences in eNOS or Akt expression in male or female kidneys.

eNOS Expression

Figure 9: (A-B) There was no statistically significant difference in eNOS expression in kidneys of female and male mice exposed to 5 ppm CdCl₂ compared to control mice (n=4/group); (p values = 0.1393 and 0.6336 respectively). [Unpaired t test]

Akt Expression
Figure 10: (A-B) There was no statistically significant difference in Akt expression in kidneys of female and male mice exposed to 5 ppm CdCl$_2$ compared to control mice (n=4/group); (p values = 0.1567 and 0.0781 respectively). [Unpaired t test]

3. Discussion of Research Findings

Most notably, and to our knowledge, this is the first study to show that cadmium exposure results in a reduction in ejection fraction and fractional shortening in males. In addition to this novel finding, this study also showed consistent results with those observed in prior studies.

First, cadmium did not alter body weight in both sexes after an 8-week exposure. As expected, there was weight gain over time in all three cohorts, because these mice arrived at 7 weeks old and were still growing. Considering weight gain or loss is known to have an effect on the cardiovascular system, eliminating this factor may be interpreted as eliminating a potential confounding variable in the other analysis within this study.
Furthermore, there was no observed difference in water consumption between the experimental and control groups across both sexes. A difference in the experimental group may have signified renal dysfunction, a metabolic disorder, or simply a developed taste aversion to the high concentration of CdCl$_2$ in the water. Nevertheless, such physiological and psychological responses may have occurred, but our study did not capture them through this measure of water consumption. Similarly, there was no observed differences in food consumption between the experimental and control groups across both sexes.

Additionally, there was no significant difference in systolic blood pressure over time between the experimental and control groups. The slight downward trend observed in both sexes from 0 to 3 weeks can be attributed to a failure of the mice being fully acclimated to the blood pressure measurement protocol. Although the mice were repeatedly trained on the protocol the week prior, perhaps more training time was necessary in order to have fully eliminated this stress effect. However, literature suggests an association between cadmium exposure and the development of hypertension, but this was not observed in the present study. This association has been based largely upon epidemiological studies and is lacking enough confirmation with animal and mechanistic studies (Eum et al., 2008; Franceschini et al., 2017; Swaddiwudhipong et al., 2010; Perry et al. 1955). The results from the present study were consistent with a previous animal study that reported no significant change in systolic blood pressure over a 6-week exposure period to CdCl$_2$ in C57BL/6J mice (Turkcan et al., 2015). With that said, a study with a longer exposure period may better recapitulate the human phenotype.
Perhaps the development of hypertension only occurs with a chronic exposure to cadmium over a period of many months or years rather than weeks.

Furthermore, analysis of data collected from echocardiography captured differences in myocardial structure and function with exposure to cadmium. Of greatest significance were the results obtained by echocardiography that showed alterations in the function of the left ventricle during contraction in males with exposure to cadmium. There is a statistically significant difference in ejection fraction and fractional shortening in the experimental group compared to the control group (p values = 0.0056 and 0.0036 respectively). Ejection fraction represents the volume of blood each cardiac contraction forces out of the left ventricle, while fractional shortening represents the shortening of the left ventricle during each cardiac contraction. For reference, an ejection fraction of 40% or lower is characteristic of heart failure in humans (Murphy et al., 2020). Although exposure to cadmium for 8 weeks was only associated with a less than a ten percent decline in ejection fraction in males, the downward trend is clear and was projected to continue downward if the exposure were to have been extended out for a longer period of time. This same trend was observed with fractional shortening in males with exposure to cadmium compared to the controls. Considering the calculation of ejection fraction and fractional shortening are completely dependent on the change in left ventricle interior diameter from diastole to systole, closer analysis of these parameters was performed and confirmed that there was a difference in left ventricle interior diameter at both end diastole and end systole. This observed increase in left ventricle interior diameter may signify the development of dilated cardiomyopathy, which is characterized by ventricular chamber enlargement, poor contractility and systolic dysfunction (Schultheiss et al.,
These findings suggest a possible dilated cardiomyopathy phenotype, because the differences in cardiac parameters were shown to be most apparent at end-systole. With that said, further experimentation would be necessary to confirm this phenotype. Specifically, more direct measures of contractility would be valuable, given ejection fraction and fractional shortening are only indirect measures of contractility. With the hearts currently stored for molecular analysis from cohort 2 in -80 °C, our lab team can measure for differences in expression of genes and proteins that are believed to be affected in hearts with dilated cardiomyopathy. These would include MYH7/ β-myosin heavy chain, TPM1/ α-tropomyosin, TNNT2/ cardiac troponin T, and TTN/ titin (McNally & Mestroni, 2017). Additionally, our lab team can measure for changes in the expression and activity of calcium handling proteins. This may provide greater insight into how cadmium may be disrupting calcium cycling and its role in regulating normal cardiac contraction on a cellular level.

Furthermore, this data suggested there was a physiologically significant difference in interventricular septum thickness between the experimental group and controls in males. The observed decrease was trending towards statistical significance at end-diastole (p value = 0.0626) but was statistically significant when measured at end-systole (p value = 0.0117). Interestingly, septal thinning has been associated with cardiac sarcoidosis in clinical studies, which is a phenotype characterized by the formation of inflammatory granulomas and presence of myocardial inflammation (Kim et al., 2009; Nagano et al., 2015). Inflammation of the septum tissue caused by cardiac sarcoidosis is believed to result in myocyte loss and cardiac fibrosis, and ultimately, thinning of the tissue (Nagano et al., 2015; Nureki et al., 2014). It would inappropriate to use the current study to draw
the conclusion that cadmium induces cardiac sarcoidosis, but this does serve as a potential explanation for the observed septal thinning. However, further experimentation is required. For example, our lab can conduct histopathological analysis of the heart slices already stained with picrosirius red and hematoxylin and eosin in search of inflammatory granulomas, collagen deposition and cardiomyocyte loss specifically within the septal tissue.

Additionally, no hypertrophy was observed in hearts exposed to cadmium in both females and males in this study (p values = 0.8142 and > 0.9999 respectively). This observation is based on a comparison of heart weight-to-tibia length ratios, which is a commonly used and reliable measure of hypertrophy in the heart. Although hypertrophy was not observed at the end of this 8-week study, perhaps it would have developed if the exposure were extended and the left ventricle dysfunction continued to worsen.

Moreover, this study showed a very slight difference in infarct size of the male hearts exposed to cadmium compared to the control group (p value = 0.1797). This difference was not observed in female hearts. (p value = 0.8868). This slight difference is intriguing and of potential interest, because this protective effect of cadmium against ischemia reperfusion injury has been previously observed. Perhaps this study was underpowered, and an increase in sample size would bring this effect to statistical significance. In any case, and given that males show this slight decrease in infarct size after exposure to cadmium, testing for an upregulation or a change in activity of mechanisms known to protect against ischemia reperfusion would be a logical next step. First, eNOS expression was measured, because nitric oxide is known to mediate cardioprotection in a cGMP dependent manner (Inserte & Garcia-Dorado, 2015). Akt was
also measured, because Akt is a serine/threonine protein kinase that is known to activate eNOS by way of phosphorylation (Dimmeler et al., 1999). Changes in the expression of these proteins were measured in kidney tissue as a surrogate for the heart because that is the tissue that was available at that point in the experiment. Although there seems to be different trends in both sexes, an increase in sample size as well as a measure of phosphorylated (activated) eNOS is necessary before any conclusions are made. These western blots also need to be repeated in cardiac tissue.

Another cardioprotective mechanism of interest includes the upregulation of metallothionein and its ability to protect against ischemia reperfusion injury. It likely confers this protection through its ability to scavenge free radicals and reduce oxidative stress (Kang, 2003; Kang, 2001; Sabolic et al., 2010). Specifically, the zinc ions form a complex with metallothionein and, during oxidative stress, the zinc is released from the complex, and the free zinc binds to apo-MTF-1 to drive increased expression of the metallothionein genes (Sabolic et al., 2010). The metallothionein protein then functions as an antioxidant by scavenging free radicals and releasing bound glutathione (Sabolic et al., 2010). Prior studies have indicated that cadmium has a higher affinity to metallothionine than essential and, therefore, is capable of displacing these ions (Hamer, 1986; Nielson et al., 1985; Sabolic et al., 2010; Waalkes & Poirier, 1984). As depicted in the figure, Sabolic et al. reviews multiple proposed methods of how cadmium causes an upregulation in the metallothionein protein. Of particular interest is the idea that cadmium causes an increase in the zinc pool and, in turn, causes increased activation of MTF-1 and, ultimately, increased production of the metallothionein protein (Sabolic et al., 2010).
Although the underlying mechanism has not been fully elucidated, cadmium exposure has been shown to induce metallothionein production in rats. Specifically, a study showed that intraperitoneal injection of cadmium chloride into male Wistar rats resulted in an increase in metallothionein levels in liver, kidney heart and aorta (Bobillier-Chaumont et al., 2006). Another study demonstrated male Wistar rats pretreated with cadmium chloride showed protection against the normal reduction in metallothionein levels and higher function recovery post ischemia. A study also suggested there is a dose and sex dependent effect in metallothionein induction, by reporting that only a high dose of cadmium exposure was associated with an upregulation in metallothionein in females (Liang et al., 2019). With the hearts currently stored for molecular analysis from cohort 2 in -80 °C, our lab team can measure for differences in metallothionein expression. An observed increase in expression would demonstrate that
chronic exposure to via cadmium chloride through drinking water has a similar effect after intraperitoneal injection as seen in prior experiments.

This study presents the novel finding that cadmium exposure causes a reduction in left ventricular function and septal thickness, which is of great interest. Our findings also suggest a potential protective role for cadmium against ischemia reperfusion injury. However, this difference was not statistically significant, which can be attributed to either an underpowered experiment or a null effect. Regardless, the most significant finding is certainly the downward trend in left ventricle contractility as represented by a reduction in ejection fraction and fractional shortening. This is a significant finding, because a reduction in these contractile measures is a defining characteristics of heart failure. With this new evidence strengthening the association between cadmium and CVD, further research should be conducted to better understand the underlying molecular mechanisms with simultaneous efforts aimed at reducing human exposure to cadmium.
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