Impact of Phthalates on Cardiac Function

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Dedication

To Judie, for inspiring me to help others.

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Thank you to God for guiding me along this journey. Thank you, Chaz, for your continued support, I could not pursue my dreams without you. Thank you to my family for always cheering me on! Gabby, thank you for always encouraging me and for always being willing to lend an ear. Thank you to Luke for assisting with data collection, it would have been near impossible to collect this information without you. Thank you to Devon for providing some of the background knowledge and data used in these studies. Blake, thank you for your assistance with data collection, answering all my questions, and being a great lab partner and friend. Dr. Kay and Dr. Entcheva, thank you for your willingness to serve on my committee and provide valuable feedback throughout this process. Lastly, thank you to Dr. Posnack, for your patience, guidance, and support, I continue to learn so much from your mentorship.

Abstract

Impact of Phthalates on Cardiac Function

Di(2-ethylhexyl) phthalate (DEHP) is a commonly used plasticizer additive that is combined with polyvinyl chloride (PVC) to create flexible plastics, including medical plastic devices. Since DEHP is a lipophilic chemical that is not covalently bound to PVC, it is prone to leach or migrate into blood[21]. Consequently, studies have shown that phthalates may contribute to cardiovascular mortality.[65] However, it is still unclear if phthalates directly contribute to adverse health complications, particularly among intensive care and cardiac intensive care patients who may experience arrhythmias, low cardiac output, and longer hospital stays[59] post exposure. Unfortunately, it is difficult to assess the direct cardiotoxic effects of DEHP in a clinical setting. Fortunately, experimental animal models can be used to better understand the direct effects of phthalate exposure on cardiac function. Likewise, this study aims to investigate the direct effects of DEHP exposure on cardiac electrophysiology.

In this study, animal hearts were excised, the aorta cannulated, and the tissue retrograde perfused with a crystalloid buffer. Electrophysiology (EP) studies were performed in the presence or absence of 250 μM DEHP, over the course of 60 minutes. Flow rate and electrocardiograms (ECGs) were monitored continuously. Over 60 minutes, cardiac function remained stable during control buffer perfusion (heart rate - baseline: 322 BPM, 60 min: 318 BPM, n=8; coronary flow rate - baseline: 6.19 mL/min, 60 min: 5.67 mL/min, n=8; sinus node recovery time (SNRT) – baseline: 211 ms, 60 min: 209 ms, n=9). However, 60 minutes of DEHP exposure resulted in a steep decrease in cardiac function,

with a 52% decrease in heart rate (baseline: 330 BPM, 60 min: 158 BPM) (n=10), a 63% decrease in flow rate (baseline: 4.62 mL/min, 60 min: 1.70 mL/min) (n=10), and a 53% increase in SNRT (baseline: 184 ms, 60 min: 282 ms, n=5). DEHP exposure also impaired atrioventricular (AV) conduction, with a 53% increase in the PR interval (baseline: 43 ms, 60 min: 66 ms) (n=5), and 82% increase in PR segment (baseline: 28 ms, 60 min: 51 ms) (n=5), 92% increase in Wenckebach Cycle Length (WBCL) (baseline: 87 ms, 60 min: 167 ms) (n=8), and a 106% increase in the atrioventricular node effective refractory period (AVNERP) (baseline: 79 ms, 60 min: 163 ms) (n=8). By comparison, only a slight increase in AV conduction time was observed in time control studies (PR interval; baseline: 34 ms, 60 min: 40 ms; n=8, PR segment; baseline: 20 ms, 60 min: 24 ms; n=8, WBCL; baseline: 87 ms, 60 min: 92 ms; n=10, AVNERP; baseline: 71 ms, 60 min: 75 ms; n=10). Finally, atrial action potential duration (APD) shortened after DEHP exposure, APD 30 (baseline: 22 ms, 60 min: 16 ms, baseline n=6, 60 min n=3), APD 80 (baseline: 50 ms, 60 min: 28 ms, baseline n=6, 60 min n=3). Comparatively, no changes were observed in the ventricular APD after DEHP exposure, APD 30 (baseline: 19 ms, 60 min: 19 ms, baseline n=6, 60 min n=3), APD 80 (baseline: 49 ms, 60 min: 52 ms, baseline n=6, 60 min n=3).

Studies suggest that DEHP may exert cardiotoxic effects by activating the muscarinic acetylcholine (M2) receptor, which is highly expressed in nodal and atrial cells.[24] Muscarinic agonists have been shown to decrease cardiac automaticity,[9] precipitate vasoconstriction of the coronary arteries,[46] delay AV conduction,[67] and shorten atrial action potential duration (APD).[28] Additional studies are necessary to fully interpret the mechanistic effects of DEHP of cardiac physiology, and future work will investigate the use of muscarinic antagonists to circumvent phthalate-induced cardiac depression.

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Chapter 1: Normal Heart Function

1.1 Cardiac Physiology

The cardiovascular system is an essential part of the human body. Its main responsibility is to deliver nutrients and oxygen to the body's cells. One key component of this system is the heart, which acts as a pump, delivering oxygen-rich blood to the body. The heart is comprised of four chambers: the right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV). Within the cardiovascular system deoxygenated blood travels from the head, neck, and chest through the superior vena cava as well as the trunk, viscera, and lower limbs through the inferior vena cava. Collectively the vena cava returns deoxygenated blood to the RA, and it is pumped to the RV. With contraction, blood is ejected from the RV, out the pulmonary valve, and through the pulmonary artery to the lungs. Following gas exchange, oxygenated blood returns to the heart through the LA and into the LV. With contraction, oxygenated blood is ejected through the aortic valve and the blood is dispersed throughout the body. The cardiac cycle is comprised of a period of rest or diastole, during which blood fills both ventricles simultaneously, and contraction or systole, during which blood is ejected from the heart to the lungs or body.[35]

1.2 Cardiac Electrophysiology

The pumping function of the heart is mediated and coordinated by electrical signals. These electrical signals are important in keeping each heart chamber synchronized and enabling an effective contraction. Electrical signals originate in pacemaker cells located within the sinoatrial (SA) node. Pacemaker cells spontaneously depolarize and set the hearts' intrinsic beating rate. Electrical signals propagate from the SA node to both atria and the signal

travels to the atrioventricular (AV) node. Electrical propagation slows through the AV node, providing time for the atria to contract and increase the blood volume in the ventricles. The signal then propagates to the bundle of His which divides into two main bundle branches, which each enter the RV and LV. Electrical signals propagate down the bundle branches and into the Purkinje fibers, a dense network that facilitates coordinated and synchronized activation of both the RV and LV.[35]

Atrial and ventricular cardiomyocytes have a resting membrane potential, which is established by electrical and chemical gradients. With each heartbeat, positive sodium ions propagate through gap junctions to nearby cells. This positive charge alters the membrane potential until a threshold voltage is achieved and an action potential fires. Depolarization is characterized by a steep upstroke (phase 0) as fast voltage gated sodium channels open. As the cardiomyocyte becomes more positively charged, the sodium channels close and the potassium channels open, creating a downward deflection and phase 1 of the cardiac action potential. This small deflection reduces the transmembrane potential to 0 mV, and in turn, voltage gated calcium channels open, creating phase 2 of the plateau phase of the cardiac action potential. Eventually the calcium channels become inactivated, and the potassium channels remain open while the cell repolarizes to its resting potential, -90 mV, resulting in a slower downward deflection and phase 3 of the cardiac action potential. The potassium channels remain open and maintain the transmembrane potential of the cell in its resting state, until the process is repeated.[44]

For the cardiomyocytes to communicate effectively, fast electrical propagation is needed. This fast propagation is achieved through channels, called gap junctions, located between cardiomyocytes. Gap junctions are low resistance channels that help facilitate the transfer of an action potential from one cardiomyocyte to the next.[12] Each gap junction is formed by two hemichannels that are made up of protein subunits called connexins (Cx).[12] Cx40 is a common connexin in atrial cardiomyocytes.[12] A mechanical stimulation activates inositol triphosphate (IP_3), which in turn releases intracellular calcium from the sarcoplasmic reticulum (SR).[30] The increased intracellular calcium stimulates the opening of gap junctions and an intracellular calcium wave travels through each gap junction.[30] Gap junction stimulations allows signals to propagate between cells.[12] Gap junctions are also signaled to close when a potential difference occurs between cells, to inhibit the propagation of signals between cardiomyocytes.[54] This intercellular communication is important in regulating signal propagation and ensuring stable electrical activity.

1.3 Excitation-Contraction Coupling

During the cardiac action potential, inward calcium current initiates myocyte contraction through a process called calcium induced calcium release.[5] As calcium enters the cell it interacts with ryanodine receptors on the SR, which triggers calcium release.[5] Intracellular calcium ions bind to troponin C, resulting in a conformational change that allows myosin heads to interact with actin myofilaments.[8] Actin and myosin myofilaments slide over each other in an ATP-dependent process that facilitates muscle contraction.[8]

1.4 Muscarinic Acetylcholine Receptor

Cardiac function is regulated by the sympathetic and parasympathetic nervous system. The sympathetic nervous system regulates the 'fight or flight' response, while the parasympathetic nervous system helps keep the body relaxed.[64] Parasympathetic vagal nerves innervate the atrial and nodal tissue and help regulate heart rate through the release of acetylcholine (ACh).[6] ACh acts as an agonist by binding and activating both nicotinic and muscarinic receptors.[6] The muscarinic acetylcholine (M2) receptor is the predominate muscarinic receptor located within the heart. M2 receptors are coupled with inhibitory G (G_i) protein,[20] which when stimulated[40] results in an inhibitory action of adenylate cyclase (AC) and a reduction in intracellular cyclic AMP (cAMP) levels.[64] Separately, G_i stimulation also increases IK_{ACh} current.[66] The decrease in cAMP levels and increase in IK_{ACh} both contribute to a slowed heart rate.[66] Increased IK_{ACh} current also shortens the APD of atrial myocytes by hastening repolarization. This results in a hyperpolarized resting membrane potential, causing the myocytes to become less excitable.[28] The latter can reduce atrial and nodal conduction velocity[28] and contribute to a slower heart rate.[64] Another contributor to reduced heart rate, is a reduction of cAMP.[26] cAMP helps regulate funny current (I_f) in the SA node[26]. If cAMP is reduced so is the activity of the I_f channel and in turn the electrical signals from the SA node.

Chapter 2: Phthalates

Phthalates are plasticizer additives that combine with PVC to create flexible plastics. Di(2-ethylhexyl) phthalate (DEHP) is the most used phthalate, with more than two million tons produced annually.[57] DEHP containing plastics are found in many consumer products and medical devices, including intravenous bags, medical tubing, and blood storage bags. Since DEHP is a lipophilic chemical that is not covalently bound to the PVC matrix, it is prone to leach under normal conditions of use.[57] Leaching is exacerbated when plastics encounter blood or lipophilic solutions. There is growing concern over the use of phthalates as heightened exposure has been linked to reproductive and cardiovascular toxicity. Current literature suggests phthalates could impact testicular development, reduce fertility, and play a role in endometriosis.[13] Phthalates have also been thought to influence cardiac function, leading to cardiotoxicity.[42]

2.1 Epidemiological Studies

Due to its abundant use in plastic food packaging,[39] humans are exposed to DEHP daily. Studies have shown a correlation between increased DEHP exposure and adverse health outcomes, such as reproductive[51] and cardiovascular [39] disease. Though US regulatory agencies have banned DEHP levels of more than 0.1% in children's toys,[29] its use is still highly prevalent in medical equipment, where it can make up to 80% of the product's weight.[51] A safety assessment conducted by the Food and Drug Administration (FDA) found DEHP levels in adults reached up to 72.9 mg/day following coronary artery bypass graft (CABG) and up to 167.9 mg/day in artificial heart transplant patients.[15] Further, DEHP exposure levels can reach up to 123.1 μg/mL in infants receiving red blood cell

(RBC) transfusions and up to 33.5 μg/mL after 24 days on extracorporeal membrane oxygenation (ECMO).[15] Unfortunately, epidemiological studies have linked phthalate exposure to higher blood pressure in youths along with higher coronary risk in seniors.[51] Since DEHP exposure is extremely high in cardiac patients, these suggested coronary risks are cause for concern.

2.2 Cardiotoxicity

Phthalate exposure has been linked to cardiotoxicity, impacting the viability and function of cardiomyocytes. Rubin et al. showed a drastic reduction in myocyte viability after acute DEHP exposure. In their study, beating chick embryo heart cells were exposed to 4 μg/mL DEHP. After 30 minutes, only 3 percent of the heart cells were still beating. After 24 hours, only 3 percent of the embryos were still viable, with most displaying autolysis.[58] A study from Rock et al. showed phthalates to reduce heart rate. In Rock's study, rats were injected with mono-2-ethylhexyl phthalate (MEHP), a metabolite of DEHP.[55] This study used a linear model to show 20 mg of MEHP reduced heart rate.[55] When the levels of MEHP in the blood surpassed 75 μg/mL, rats were shown to go into cardiac arrest.[55] Additionally, Jaimes et al. showed MEHP to impact cardiac electrophysiology.[27] In their study, 30 minutes of 60 μM MEHP exposure showed significant lengthening of atrioventricular conduction time when compared to control studies.[27]

Studies have also shown phthalates to have an impact on calcium and contractility. In a study by Posnack et al., human stem cell-derived cardiomyocytes (hESC-CM) were exposed to 50 μg/mL DEHP for 72 hours. The calcium transient amplitudes of these cells

were reduced by almost half, post DEHP exposure.[52] Barry et al showed MEHP to inhibit cardiac contractility.[4] In their study, when human RA trabeculae were exposed to a range of 0 - 175 μg/mL MEHP, a concentration dependent reduction in contractile force was observed.[4]

A later study by Barry et al. investigated possible mechanistic effects of phthalates on cardiomyocytes and found atropine pretreatment to reduce the effects of phthalates, suggesting they act in a similar manner as actylcholine.[3] ACh is released when the parasympathetic nervous system is activated, namely M2 receptors.[7] If phthalates act similarly to ACh, they may behave as a M2 receptor agonist. Furthermore, if DEHP acts comparably, increased M2 activity would alter intracellular cAMP signaling, along with an increase in IK_{ACh} current. Therefore, M2 receptor upregulation and several cardiotoxic effects, such as altered regulation and altered cell coupling, are anticipated.

2.2.1 Altered Cardiac Automaticity and Electrophysiology

The autonomic nervous system, which includes the sympathetic and parasympathetic nervous system, regulates heart rate and cardiac contractility.[68] When an increased release of ACh occurs, the M2 receptor activity is increased[40], and this drives a reduction in automaticity.[9] It is anticipated that DEHP may contribute to a reduction in automaticity by upregulating the M2 receptor, akin to ACh. Furthermore, altered cardiac electrophysiology is expected, as observed with ACh exposure. Such ACh effects are shown in a study by Wang et al. They showed patients exposed to ACh have a significant increase in AVNERP duration, as well as WBCL duration.[67] Additionally,

stimulation of muscarinic receptors in smooth muscle cells have been shown to cause vasoconstriction of the coronary arteries.[47] Ludmer et al. illustrates a dose dependent dilation of the coronary arteries in response to ACh.[47] Lamping et al. also showed M2 receptors in the smooth muscle cells of vascular tissue.[34] Collectively, it can be hypothesized that DEHP exposure would reduce automaticity, slow AV conduction, and reduce coronary flow.

2.2.2 Altered Cardiomyocyte Coupling

In addition to its potential effects on M2 receptors, DEHP may also interfere with gap junction channels. Gap junctions are necessary for intercellular communication and depolarization,[56] when they become uncoupled, conduction velocity is reduced.[56] Previous work has shown phthalate interference of gap junction intercellular communication (GJIC) in a variety of cell models, including cardiomyocytes[52], Sertoli cells[70], and fibroblasts[18]. An increase in the distance between gap junctions of Sertoli cells was demonstrated in a study by Yao et al., after 12-hours of exposure to MEHP.[70] Furthermore, Gillum et al. showed DEHP doses, as low as 1 μg/mL, significantly reduced conduction velocity in cardiomyocytes, after 24-hour exposure.[18] Therefore, it is hypothesized that DEHP may impact GJIC, contributing to slowed atrioventricular conduction of an intact heart model.

2.3 Specific Aim and Hypothesis

Current literature suggests phthalate chemicals may act as an agonist for muscarinic acetylcholine receptors (M2)[50] located in nodal and atrial cardiomyocytes[24], Figure 1.

When these receptors become activated, outward potassium current IK_{ACh} increases[28] and cAMP levels decrease[49]. Through this interaction, I hypothesize that DEHP will slow sinus rate and delay atrioventricular (AV) conduction. Furthermore, I hypothesize an increase in IK_{ACh} current will hasten repolarization, and because the M2 receptors are most prominent in the atria[60], I expect hastened repolarization will contribute to a shorted atrial action potential duration (APD).[19] Moreover, I hypothesize the increased IK_{ACh} will also hyperpolarize the cells, slowing phase 4 depolarization in nodal cells, and will result in a reduce heart rate.[41,60] Additionally, I hypothesize this hyperpolarization will slow atrioventricular conduction, as the slope of the action potential upstroke will be decreased.[14,60]

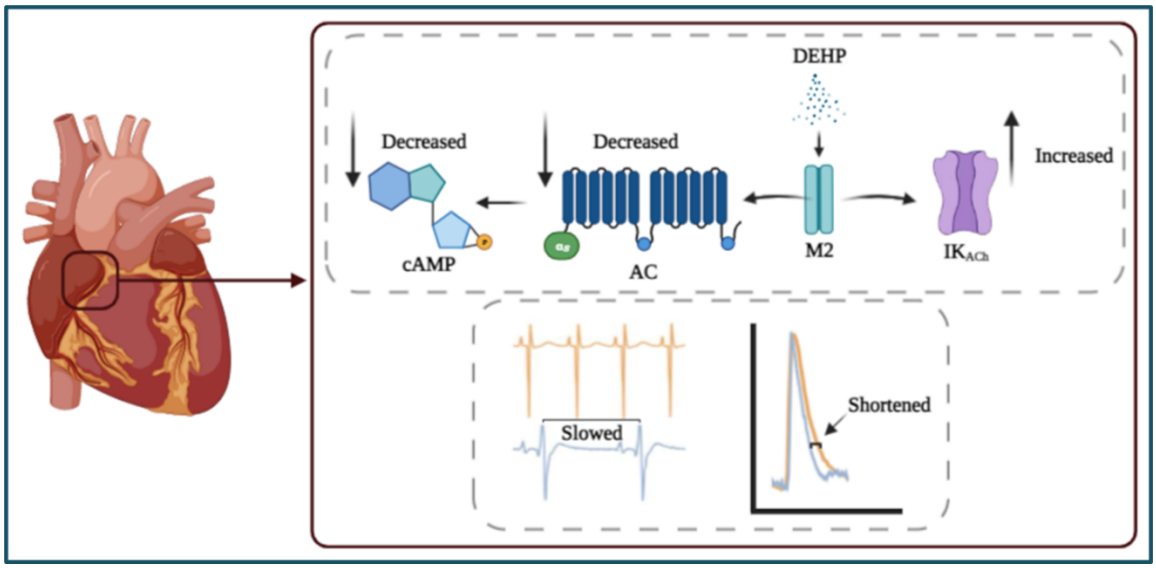

Figure 1: Hypothesized DEHP Effects. **a** DEHP activates the M2 receptor, increasing IK_{ACh} current and inhibiting adenylate cyclase (AC) activity. Inhibited AC activity lowers cAMP levels. **b** Increased IK_{ACh} shortens atrial APD. Reduced cAMP levels and increased IK_{ACh} slow heart rate or automaticity.

Chapter 3: Methods

3.1 Reagents

A crystalloid buffer, Krebs Henseleit (KH), was used to perfuse an excised intact heart preparation. One liter of the KH buffer solution included 41.4 g NaCl, 1.47 g KCl, 0.8665 g $MgSo_4$, 0.9798 g KH_2PO_4, 12.097 g $NaHCO_3$, 10.8 g Glucose, 1.32 g Na Pyruvate, 60 mL Hepes, and 0.22 g $CaCl_2$. Throughout the studies, KH buffer was bubbled with carbogen, a gas with 95% oxygen and 5% carbon, and circulated in a 37º Celsius (C) temperature-controlled system.

Ice cold cardioplegia was used to stop cardiac contraction during the cannulation process. Cardioplegia was made by supplementing 1 L KH buffer with 1.11 g of KCl. Cardioplegia was added to preparation dishes and the intact hearts were submerged after excision.

A 1 mg stock of blebbistatin (Apex Bio or Cayman) was combined with 400 µL of DMSO, resulting in a concentration of 42.7mM. 12.5 mg/mL (EP) and 37.5 mg/mL (imaging) of blebbistatin was diluted in 50 mL warmed KH, and vigorously stirred to avoid crystallization.[62] Blebbistatin acts as a mechanical uncoupler, preserving electrical activity while simultaneously preventing mechanical contraction of the heart.[62] Blebbistatin exposure stops motion that may distort imaging and may displace pacing and recording electrodes. Blebbistatin also reduces cardiac metabolic demand[63], enabling reduced oxygen and glucose consumption. At the beginning of each study, blebbistatin was added to the upper reservoir of the Langendorff perfusion system, and cardiac contraction ceased

approximately 10 minutes after perfusion. The final working concentration of blebbistatin was 4.27 μM (EP) and 12.81 μM (imaging).

A voltage dye, RH 237 (Apex Bio), was used to collect the transmembrane voltage signals of the heart. 5 mg of RH 237 was combined with 4 mL of DMSO, with a resulting concentration of 2.5 mM. 1.2 μg/μL of RH 237 was vortexed with 1 mL KH buffer, with a final concentration of 126 μM. Care was taken to remove air bubbles from the dye, before injecting into the aortic cannula, to prevent an air embolism.

50 μL of DEHP was combined with 0.5 mL of DMSO and added to the upper reservoir of the Langendorff system, resulting in a circulating concentration of 250 μM DEHP in 500 mL of KH buffer. This concentration was equivalent to clinical exposure after blood transfusion, as defined in Table 1 (unpublished data, Devon Guerrelli). Table 1 shows DEHP concentrations in blood bags ranging from 60-282 μM. The dosage used for these studies was comparable to the upper range of DEHP levels in blood bags after 40 days of storage.

Blood Bag DEHP Levels	
Days Stored	**DEHP µM (range)**
Day 7	60 - 88
Day 16	76 - 107
Day 30	119 - 149
Day 35	133 - 196
Day 40	108 - 282

Table 1: Phthalate Exposure in a Clinical Setting. DEHP levels ranging from 60 µM to 282 µM in stored blood bags (unpublished data, Devon Guerrelli).

3.2 Animals

Experiments were conducted with male and female Sprague-Dawley rats, aged 6-8 weeks, weighing 228 g ± 75 g (Taconic Biosciences, strain NTac:SD, from NIH Genetic Resource stock, n=23). The animal protocols used were approved The Institutional Animal Care and Use Committee at Children's National Research Institute. The animals were housed in the Research Animal Facility, in acrylic rat cages, and under standard environmental conditions (12:12 hour light:dark cycle, 18-25º C, 30-70% humidity).[53]

3.3 Surgery

3.3.1 Anesthesia

The animals underwent deep anesthesia in an induction chamber, supplied with 2% isoflurane for 20 minutes. Before surgery, the animal was placed on its back and transferred to a nose cone, where anesthesia was maintained with 3% isoflurane. The surgical depth of the animal was tested with a standard foot and tail pinch. 0.5 mL of heparin was then injected intraperitoneally into the animal as an anticoagulant, minimizing the possibility of blood clots during heart isolation.

3.3.2 Heart Isolation

The xiphoid process was located and the skin above it removed, exposing the subcutaneous tissue. Two incisions were made toward both armpits to expose the diaphragm. The xiphoid process was pulled superiorly, and the diaphragm opened, allowing for surgical access to the heart tissue. The heart was excised from the cavity and quickly submerged in ice cold cardioplegia.

Cardioplegia was used to halt electrical, mechanical, and metabolic activity of the heart, which helped to preserve the tissue during cannulation. The aorta was located, and forceps were used to guide the aorta onto a cannula connected to a syringe with KH buffer. Warmed KH was then retrograde perfused through the heart until the coronary arteries were cleared. The heart was transferred to a Langendorff system, and continuously perfused with oxygenated 37º C KH buffer.

3.4 The Langendorff System

Oskar Langendorff developed the concept of retrograde perfusion with mammalian hearts in 1895, eventually known as Langendorff perfusion.[37] This method of perfusion causes the aortic valves to close and the solution to perfuse the coronary arteries. A constant pressure Langendorff system was used rather than a constant flow setup, as DEHP was hypothesized to impact flow rate. This setup allowed continuous monitoring of the coronary flow rate throughout the study.

Figure 2 demonstrates the components of a Langendorff perfusion system. First, the KH buffer was added to the lower reservoir and bubbled with carbogen (a 95% oxygen, 5% carbon dioxide mixture). The KH buffer was pumped through a 12μM filter and into a heating chamber, which warmed the buffer to a normal body temperature of 37º C. The KH buffer was then pumped to a reservoir at a set height, creating 60-70 mmHg of hydrostatic pressure, and was bubbled with carbogen, to ensure adequate oxygenation. The KH buffer then flowed through a bubble trap, to prevent air from entering the heart, and retrograde perfused the heart tissue at its intrinsic flow rate.

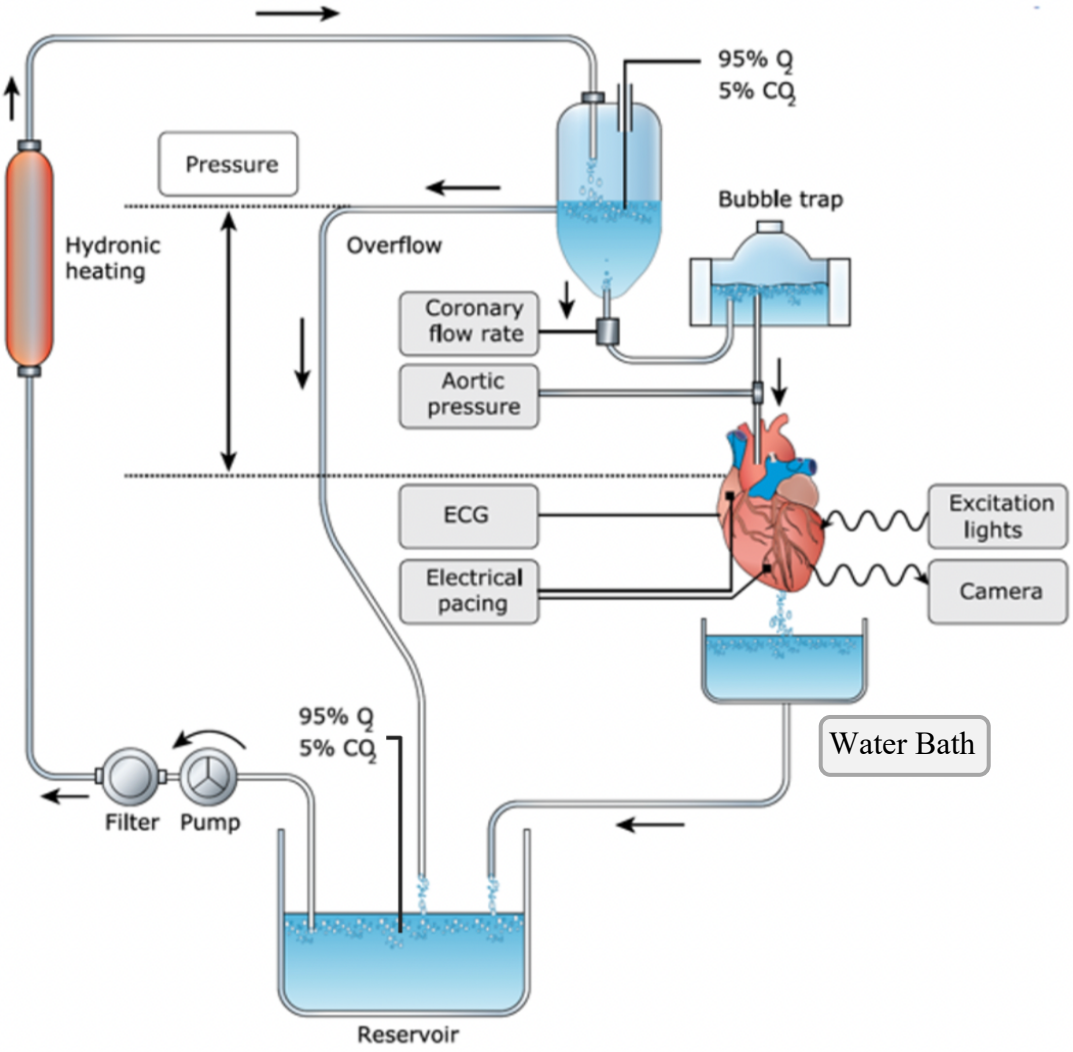

Figure 2: Langendorff System. Edited from Swift et al. 2019[61] (explicit permission from JoVE shown in Appendix I). KH buffer was bubbled in the lower reservoir, pumped through a filter, and heated with the hydronic heating system. It flowed to the upper reservoir, was bubbled, and traveled through a bubble trap before it retrograde perfused the heart. Overflow from the upper reservoir and bath were pumped to the bottom reservoir.

3.5 Electrophysiology Techniques

3.5.1 Electrocardiogram

Electrodes were placed in a lead I configuration, near the left and right atrium, to continuously record ECGs. Biosignals were continuously acquired throughout the study using ADInstruments LabChart. Under normal sinus rhythm, ECGs were used to compute heart rate, PR interval, and PR segment. The ECGs were also used to pinpoint the ventricular refractory period (VERP), Wenckebach cycle length (WBCL), atrioventricular refractory period (AVNERP), and sinus node recovery time (SNRT) in response to external pacing.

3.5.2 Pacing the Heart

The VERP was determined by pacing the apex of the LV using an S1-S2 pacing protocol, with eight beats (S1) at a 150 ms interval and a ninth beat (S2) decremented from a 140 ms interval until S2 failed to capture. Wenckebach cycle length (WBCL) was determined by pacing the RA using an S1-S1 pacing protocol, with eight beats (S1) decremented from a 150 ms interval until a beat failed to capture. Atrioventricular refractory period (AVNERP) was determined by pacing the RA using an S1-S2 pacing protocol, with eight beats (S1) at a 150 ms interval and a ninth beat (S2) decremented from a 140 ms interval until S2 failed to capture. Sinus node recovery time (SNRT) was determined by pacing the RA at a 150 ms interval for 20 beats and calculating the distance between the final paced beat and the following intrinsic beat.

3.6 Imaging

For all imaging studies, burst pacing at 200 ms, 150 ms, and 100 ms was used to pace the apex of the LV. Optical mapping allowed the transmembrane voltage to be measured and analyzed, with 450 frames acquired at 400 frames per second (FPS) using a pco.dimax camera during burst pacing. This process was repeated after 60 minutes with or without exposure to DEHP. A MATLAB program was used to analyze the atrial and ventricular action potential from both the RA and LV.

3.6.1 Imaging Set Up

Figure 3 demonstrates the imaging set up used. Two LED lights emitted a wavelength of 525nm ± 20nm, which excited the RH 237 voltage dye, with an excitation peak at 550 nm. RH 237 responds to changes in transmembrane potential by altering its spectral properties. When cells depolarize, the dye's peak emission (786 nm) shifts to a shorter wavelength and vice versa when the cells repolarize (figure 3c-d).[33] A 710 nm long pass filter was used to collect emission above 710 nm, as shown in Figure 3b. The emitted light was captured with a pco.dimax camera (Figure 3a) and the voltage signals from the image were analyzed.

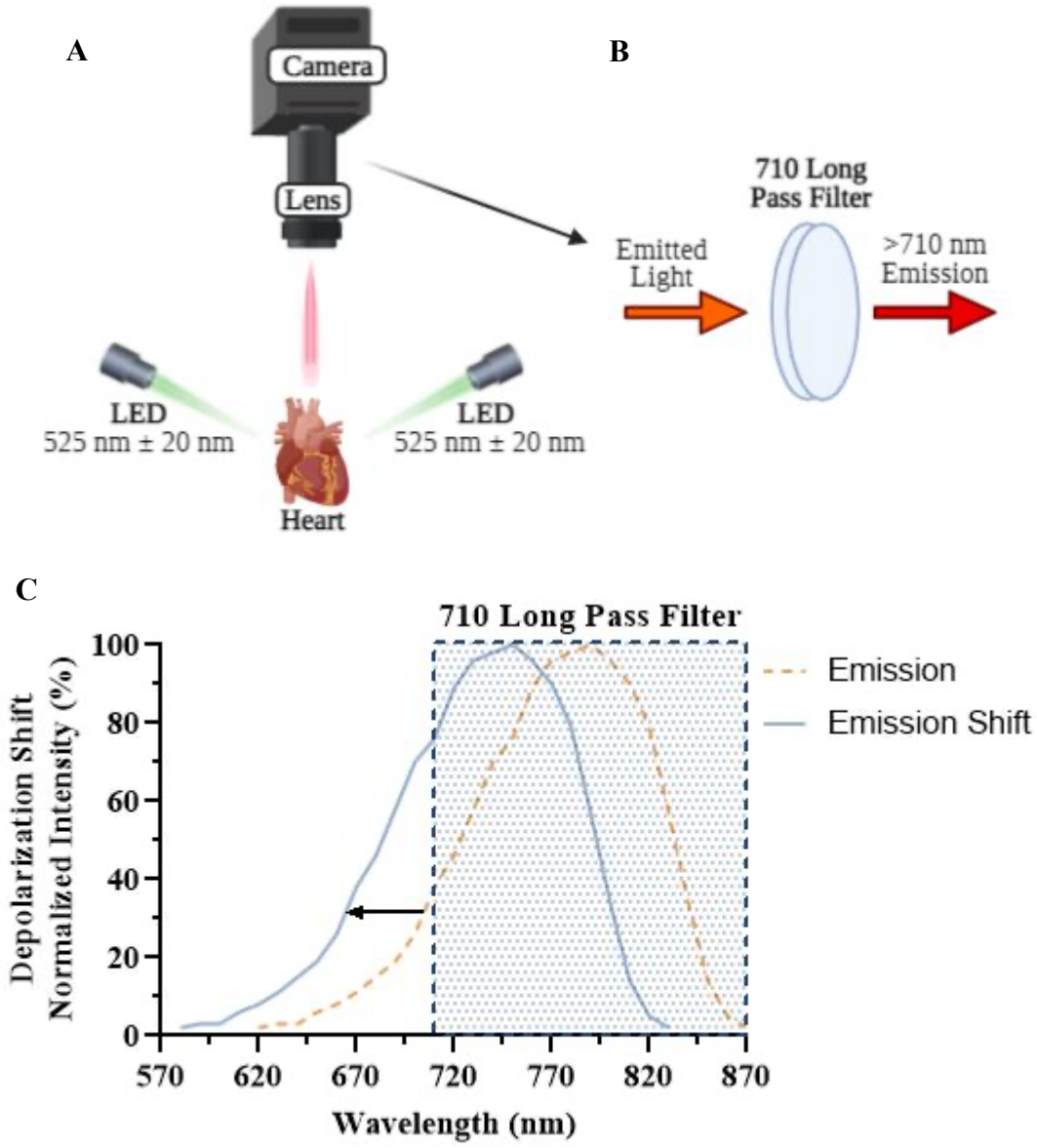

Figure 3: Imaging Set Up. **a** LEDs excite the RH 237, in the heart, at 525 nm. A lens directs the emitted light to a pco.dimax camera. **b** A 710 nm long pass filter was used to collect RH 237 emission. **c** RH 237 emission depolarization shift with 710 long pass filter.

3.7 Statistical Analysis

Parametric two-tailed paired t-test analysis was used to compare baseline measurements to 60-minute measurements in control or DEHP exposure studies. Significance was defined as $p < 0.05$. The bar graphs show a mean, with each point representing a replicate, and each line signifying the standard deviation (SD). The line graphs show a mean, with each replicate denoted as a dot, and each vertical line signifying the SD. A minimum of three APD measurements were analyzed and averaged at each time point, following control or DEHP exposure. One AVNERP and one WBCL parameter was used at each time point, per heart, while two SNRT measurements were averaged at each time point, per heart. Heart rate, flow, and ECG time course analysis was conducted every 10 minutes, with a minimum of 50 beats for each ECG analysis. Baseline recordings were measured after the heart stabilized and dyes were added (approximately 45 minutes), and recordings were measured again one hour after continued control buffer perfusion or DEHP exposure (approximately 105 minutes from start of study). R^2 values were reported for the time course studies.

Chapter 4: Results

4.1 Reduced Automaticity

Previous studies have shown reduced automaticity from exposure to MEHP.[27] Accordingly, ECG traces were monitored in Langendorff perfused hearts with and without exposure to 250 μM DEHP, to determine the effect DEHP has on automaticity. The effect of time and DEHP on heart rate can be seen in Figure 4 below. SNRT measurements were also taken at baseline and after 60 minutes with or without exposure to DEHP. The effect of DEHP on SNRT duration can be seen in Figure 5 below. The results from both Figures 4 and 5 demonstrate the minimal effect of time on Langendorff perfused hearts and the significant effect DEHP has on reducing cardiac automaticity.

4.1.1 Heart Rate

Control studies maintained an average heart rate of 322 BPM, over the course of 60 minutes (Figure 4c) with minimal variation (baseline: 322 BPM, 60 min: 318 BPM, baseline SD=33 BPM, 60 min SD=31 BPM, n=8), a sample trace of control ECGs is shown in Figure 4a. On the contrary, hearts exposed to DEHP for 60 minutes showed significantly reduced heart rates ($p<.001$), with over a 50% reduction, dropping from 330 BPM to an average of 158 BPM (baseline: 330 BPM, 60 min: 158 BPM, baseline SD=40 BPM, 60 min SD=88 BPM, n=10), as shown in Figure 4b-c. Over 165 minutes, control studies continued to show little change in heart rate ($R^2=.037$). However, DEHP exposed hearts showed a steep decline in heart rate ($R^2 =.599$). Both are shown in Figure 4d-e.

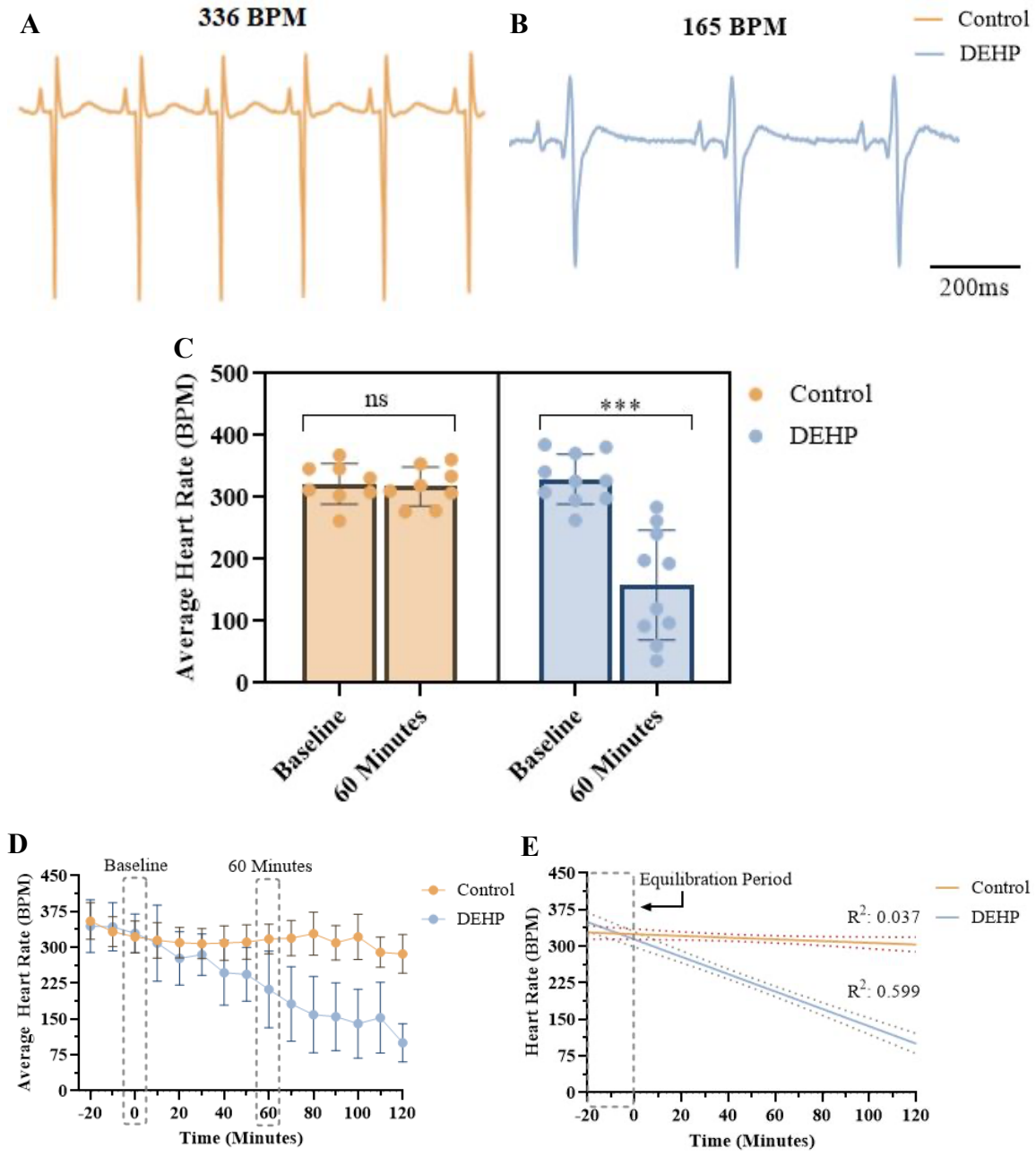

Figure 4: Heart Rate. **a** Average control ECG. **b** Average DEHP ECG. **c** Average control heart rate at baseline and 60 minutes vs. average DEHP heart rate at baseline and 60 minutes (***p<.001). **d** Average control (n=7) vs. average DEHP (n=10). **e** Simple linear regression of average heart rate for control (n=7) and DEHP (n=10) values.

4.1.2 Sinus Node Recovery Time

Sinus node conduction can be inhibited through vagal stimulation.[1] DEHP is thought to stimulate the cholinergic receptor[3], which is regulated by the vagus nerve[48], therefore SNRT protocol was conducted to determine if DEHP inhibits sinus node automaticity. SNRT measurements were found to significantly increase ($p<.01$) after one hour exposure to DEHP, increasing from 184 ms to 282 ms (baseline: 184 ms, 60 min: 282 ms, baseline SD=21 ms, 60 min SD=58 ms, n=5), while control studies showed no significant increase, with the duration time steady around 210 ms (baseline: 211 ms, 60 min: 209 ms, baseline SD=23 ms, 60 min SD=14 ms, n=9). These values can be seen in Figure 5c.

The pacing protocol used is illustrated in Figure 5a-b. The RA was paced for 20 beats at 200 pacing cycle length (PCL) and the time between the final pacing beat and the intrinsic sinus rhythm were measured, resulting in the SNRT duration. Figure 5a-b gives a visual of the delay between the control (Figure 5a), and the DEHP exposed hearts (Figure 5b). The recovery time increased an average of 98 ms in DEHP exposed hearts, suggesting inhibition of sinus node function after DEHP-treatment.

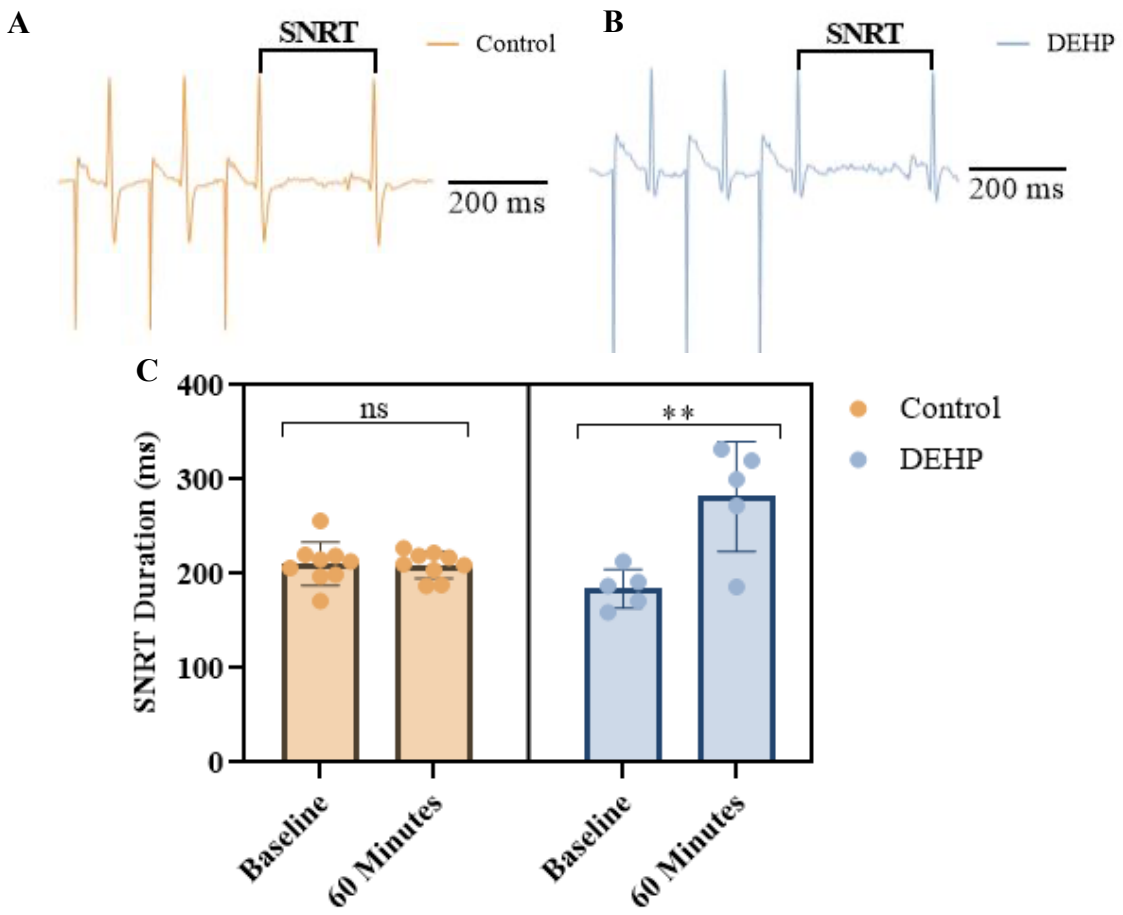

Figure 5: Sinus Node Recovery Time. **a** Control ECG trace of SNRT pacing at 150 PCL. **b** DEHP ECG trace of SNRT pacing at 150 PCL **c** Average control SNRT at baseline and 60 minutes vs. average DEHP SNRT at baseline and 60 minutes (**p<.01).

4.2 Coronary Vasoconstriction

Muscarinic agonists can cause coronary vasoconstriction and reduce coronary flow.[2] Since DEHP is thought to act in a similar manner as ACh, coronary vasoconstriction and reduced coronary flow was anticipated. Coronary flow rate was monitored with and without exposure to 250 μM DEHP, as shown in Figure 6b. Baseline and 60 minutes time points were also observed with and without exposure to DEHP, these results can be seen in Figure 6a below.

4.2.1 Coronary Flow Rate

As expected with blebbistatin pre-treatment, oxygen demand and coronary flow rate declined modestly but steadily throughout the study with control buffer perfusion,[63] dropping from 6.19 mL/min to 5.67 mL/min (baseline: 6.19 mL/min, 60 min: 5.67 mL/min, baseline SD=1.8 mL/min, 60 min SD=1.3 mL/min, $p<.05$, n=8). However, this significance is minimal when compared to the results of perfused hearts ($p<.0001$) exposed to DEHP for 60 minutes, whose flow dropped from 4.62 mL/min to 1.70 mL/min (baseline: 4.62 mL/min, 60 min: 1.70 mL/min, baseline SD=1.6 mL/min, 60 min SD=0.6 mL/min, n=10), as shown in Figure 6a. Over the course of the study, the coronary flow rate of DEHP exposed hearts dropped to below 1 mL/min ($R^2=.596$) while control hearts stayed above 5 mL/min ($R^2=.114$), shown in Figure 6b-c. These results suggest exposure time to DEHP had a larger effect on the coronary flow rate than perfusion time alone.

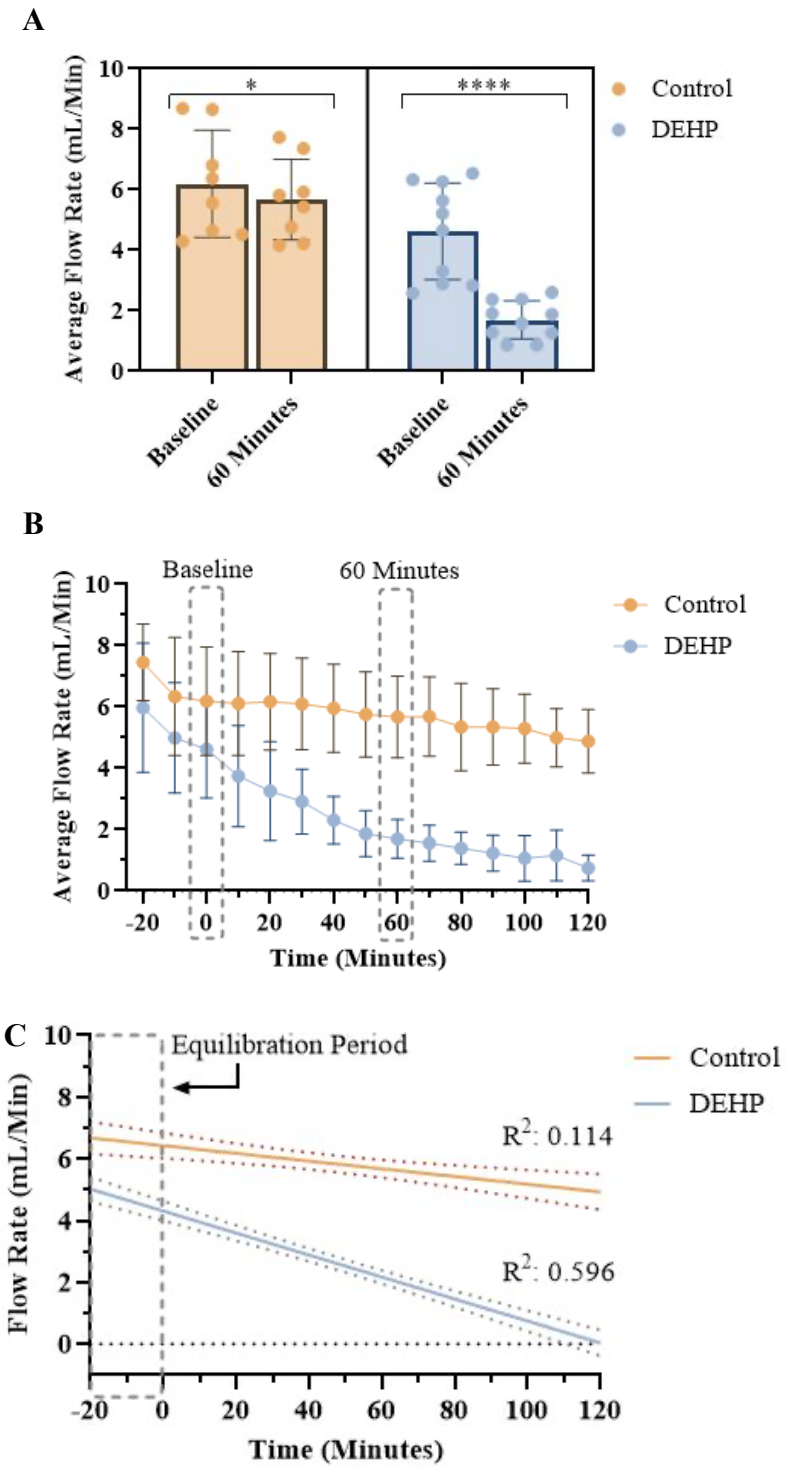

Figure 6: Flow Rate. **a** Average control flow rate at baseline and 60 minutes (*p<.05) vs. average DEHP flow rate at baseline and 60 minutes (****p<.0001). **b** Average control (n=7) vs. average DEHP (n=10) flow rate. **c** Simple linear regression of average flow rate for control (n=7) and DEHP (n=10) values.

4.3 Electrical Conduction

Previous studies suggest DEHP slows cardiac electrical conduction.[18] Accordingly, PR interval/segment parameters were measured and WBCL measurements were calculated to determine AV conduction. AVNERP measurements were also calculated to determine AV effective refractory period (ERP) duration. The control studies showed no significant increase in electrical conduction parameters, with stable PR interval/segment, WBCL, and AVNERP durations, while DEHP exposed hearts showed significant changes in these same parameters.

4.3.1 PR Interval and PR Segment

Studies have shown increased acetylcholine levels can lead to AV block.[16] AV block can be represented by a lengthening of the PR interval and PR segment.[32] Thus, PR interval and PR segment durations were observed over 165 minutes with and without DEHP exposure. PR intervals were measured from the start of the P wave to the start of the QRS complex, while PR segments were measured from the end of the P wave to the start of the QRS complex. An example of these measurements can be seen in Figure 7a, with an ECG from a DEHP exposed heart.

Control studies showed no significant increase in PR interval and PR segment after 60 minutes, increasing from 34 ms to 40 ms (baseline: 34 ms, 60 min: 40 ms, baseline SD=4 ms, 60 min SD=8 ms, n=8) and 20 ms to 24 ms (baseline: 20 ms, 60 min: 24 ms, baseline SD=5 ms, 60 min SD=8 ms, n=8) respectively, shown in Figure 7b-c. However, DEHP exposed hearts showed significant increases in PR interval ($P<.05$)

and PR segment ($p<.05$), with the PR interval increasing 53%, from 43 ms to 65 ms (baseline: 43 ms, 60 min: 66 ms, baseline SD=4 ms, 60 min SD=20 ms, n=5) and PR segment increasing 82%, from 28 ms to 15 ms (baseline: 28 ms, 60 min: 51 ms, baseline SD=5 ms, 60 min SD=18 ms, n=5), after one-hour DEHP exposure. These results can be seen in in Figure 7b-c.

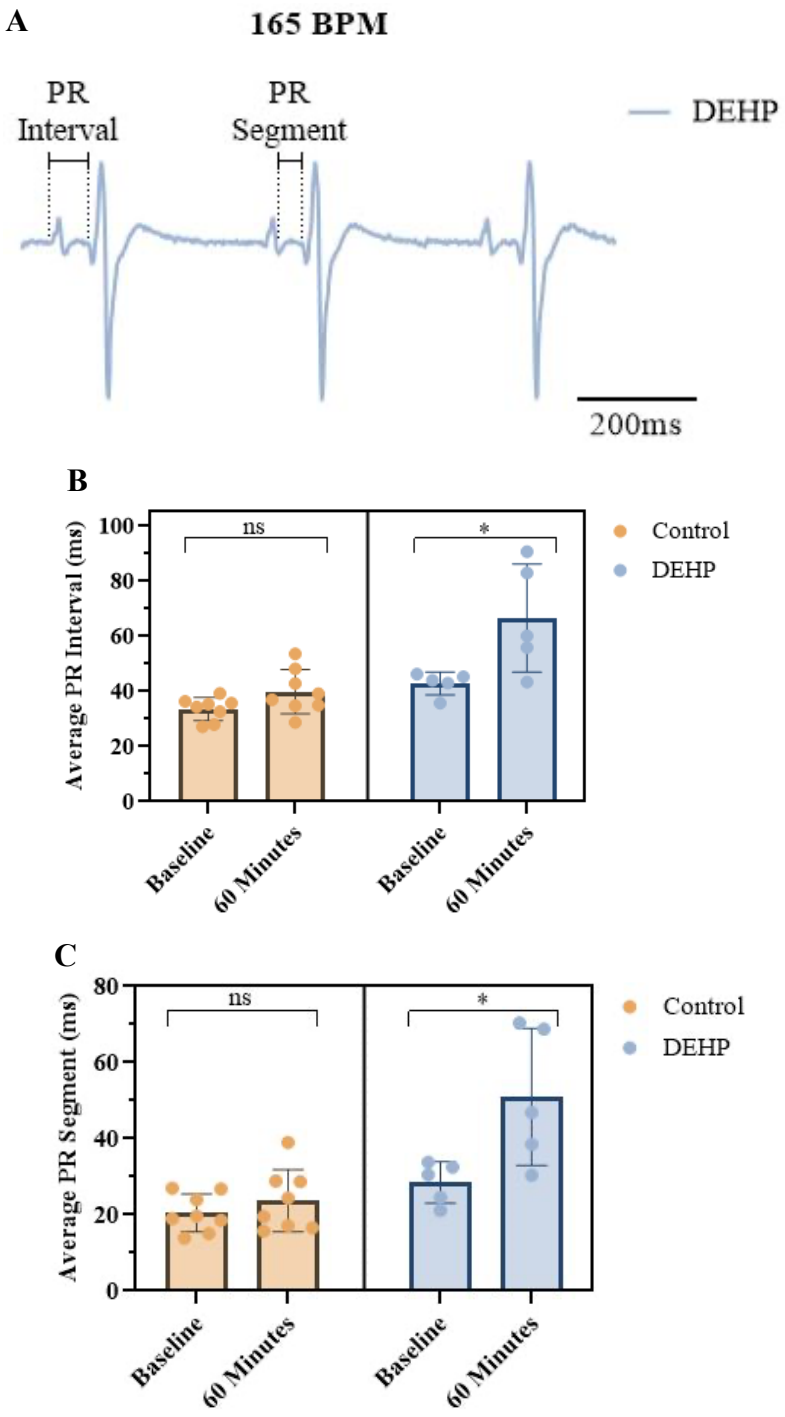

Figure 7: PR Interval and PR Segment. **a** Illustration for PR interval and PR segment calculation. **b** Average control PR interval at baseline and 60 minutes vs. average DEHP PR interval at baseline and 60 minutes (*p<.05). **c** Average control PR segment at baseline and 60 minutes vs. average DEHP PR segment at baseline and 60 minutes (*p<.05).

4.3.2 Wenckebach Cycle Length

WBCL is the longest cycle length that Wenckebach block occurs in the AV node.[71] This occurs when a consistent cycle length paces the RA, conduction through the AV node slows, and the impulse fails to conduct to the ventricles.[25] Increased vagal stimulation has been shown to increase the cycle length in which WBCL block occurs.[43] DEHP may act as a vagal stimulant by acting on the M2 receptor,[51] so an increased WBCL cycle length with DEHP exposure is hypothesized. Both control and DEHP exposed hearts underwent WBCL protocol, pacing the RA for 8 beats and incrementally reducing the PCL from 150 until a dropped beat was observed. An example of this can be seen in Figure 9a below, with the control study capturing all 8 beats and the DEHP study dropping the 7th beat, thereby failing to capture.

Control studies showed no significant increase in the WBCL duration, (baseline: 87 ms, 60 min: 92 ms, baseline SD=8 ms, 60 min SD=9 ms, n=10). However, DEHP exposed hearts showed a significant increase in WBCL duration (p=.001), increasing from 87 ms to 167 ms (baseline: 87 ms, 60 min: 167 ms, baseline SD=16 ms, 60 min SD=47 ms, n=8). These results are shown in Figure 9b below. WBCL phenomenon occurs when AV conduction has slowed and an impulse results in the QRS complex failing to follow the P wave.[38] This dropped beat allows the AV node time to recover.[38] These results suggest slowed conduction through the AV node with DEHP exposure, as the AV node was unable to repolarize as quickly.

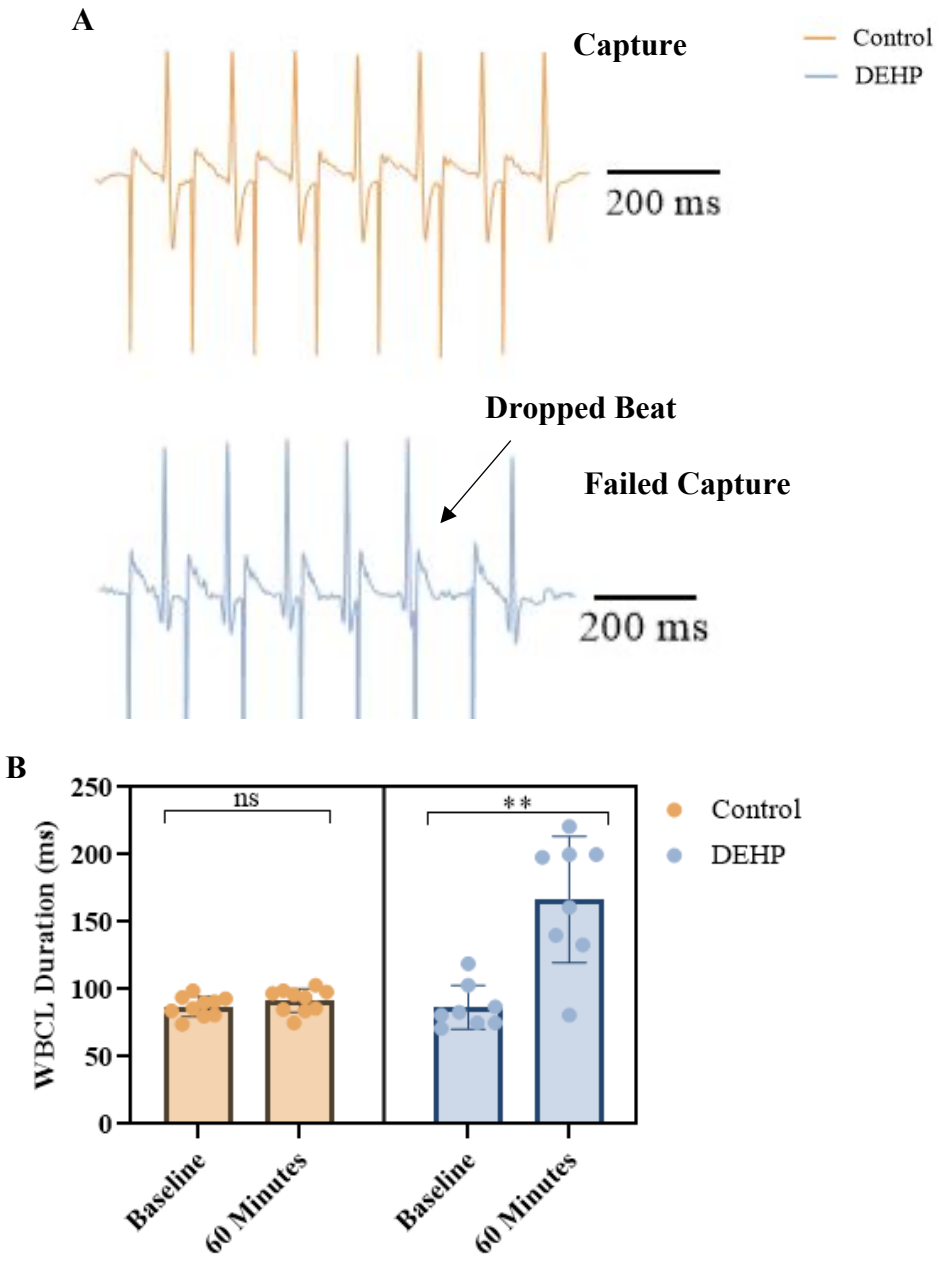

Figure 8: Wenckebach Cycle Length. **a** Illustration for WBCL measurement. **b** Average control WBCL at baseline and 60 minutes vs. average DEHP Wenckebach at baseline and 60 minutes (**p=.001).

4.3.3 Atrioventricular Refractory Period Lengthening

AVNERP is the longest S1-S2 PCL that leads to S2 not propagating through the AV node to the bundle of HIS.[17] This is also known as the ERP of the AV node.[10] An AVNERP pacing protocol was conducted to determine the ERP of the AV node. The RA was paced with S1 set to 8 beats at 150 PCL and S2 incrementally reduced from 140 until it failed to capture. Control studies showed no significant lengthening over the course of 60 minutes, (baseline: 71 ms, 60 min: 75 ms, baseline SD=6 ms, 60 min SD=8 ms, n=10). However, DEHP exposed hearts did show significant AVNERP lengthening ($p<.001$), increasing from 79 ms to 163 ms (baseline: 79 ms, 60 min: 163 ms, baseline SD=34 ms, 60 min SD=42 ms, n=8). These results can be seen in Figure 8b. Figure 8a shows the control beat capturing with S2 at 100 PCL while the DEHP exposed heart failed to capture with a similar S2. Slowed conduction through the AV node can lead to AVNERP lengthening.[10] Therefore, these results also suggest slowed AV conduction with DEHP exposure.

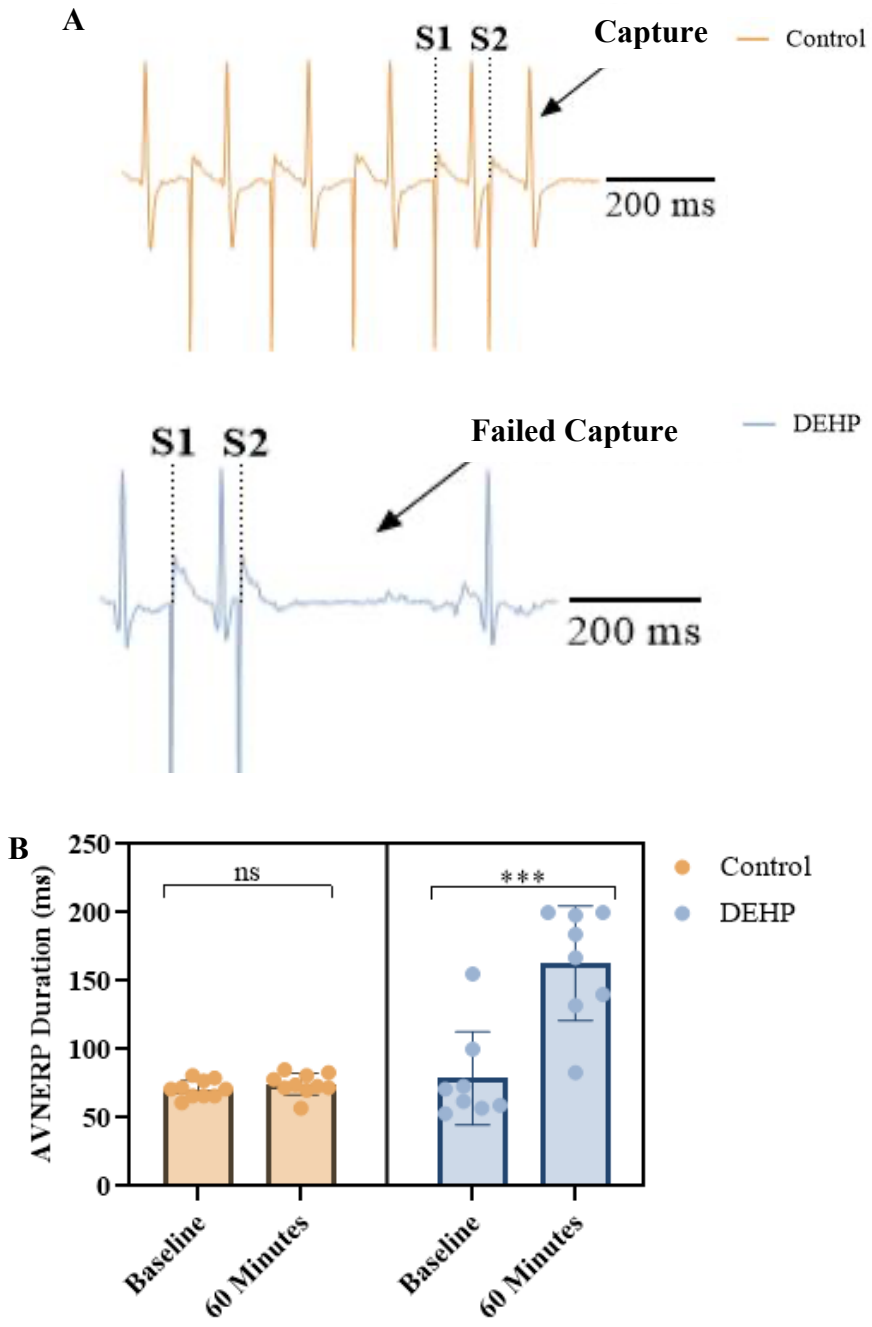

Figure 9: Atrioventricular Node Effective Refractory Period **a** Illustration for AVNERP measurement. **b** Average control Atrioventricular Node Effective Refractory Period (AVNERP) at baseline and 60 minutes vs. average DEHP AVNERP at baseline and 60 minutes (***p<.001).

4.4 Cardiac Action Potential

Atrial APD shortening has been demonstrated in response to a muscarinic agonist.[69] Since more M2 receptors are in the atria than the ventricle, [24] APD shortening is hypothesized to occur in the atria and not the ventricle with exposure to DEHP. To study this, the ventricle was paced at 200 PCL and voltage signals were collected. The APD were then analyzed from both the atria and the ventricle, with and without DEHP exposure. Figure 9a shows atrial APD shortening when exposed to DEHP compared to a baseline APD trace.

Significant atrial APD shortening was observed in both APD 30 (APD at 30 percent repolarization) and APD 80 (APD at 80 percent repolarization), shortening from 22 ms to 16 ms (baseline: 22 ms, 60 min: 16 ms, baseline SD=4 ms, 60 min SD=1 ms, baseline n=6, 60 min n=3, $p<.05$) and 50 ms to 28 ms (baseline: 50 ms, 60 min: 28 ms, baseline SD=8 ms, 60 min SD=4 ms, baseline n=6, 60 min n=3, $p<.01$), respectively. These results can be seen in Figures 9b-c. However, there was no observed APD shortening in the ventricle, both APD 30 (baseline: 19 ms, 60 min: 19 ms, baseline SD=3 ms, 60 min SD=2 ms, baseline n=6, 60 min n=3) and APD 80 (baseline: 49 ms, 60 min: 52 ms, baseline SD=8 ms, 60 min SD=7 ms, baseline n=6, 60 min n=3) show no significant change, as shown in Figure 9b-c.

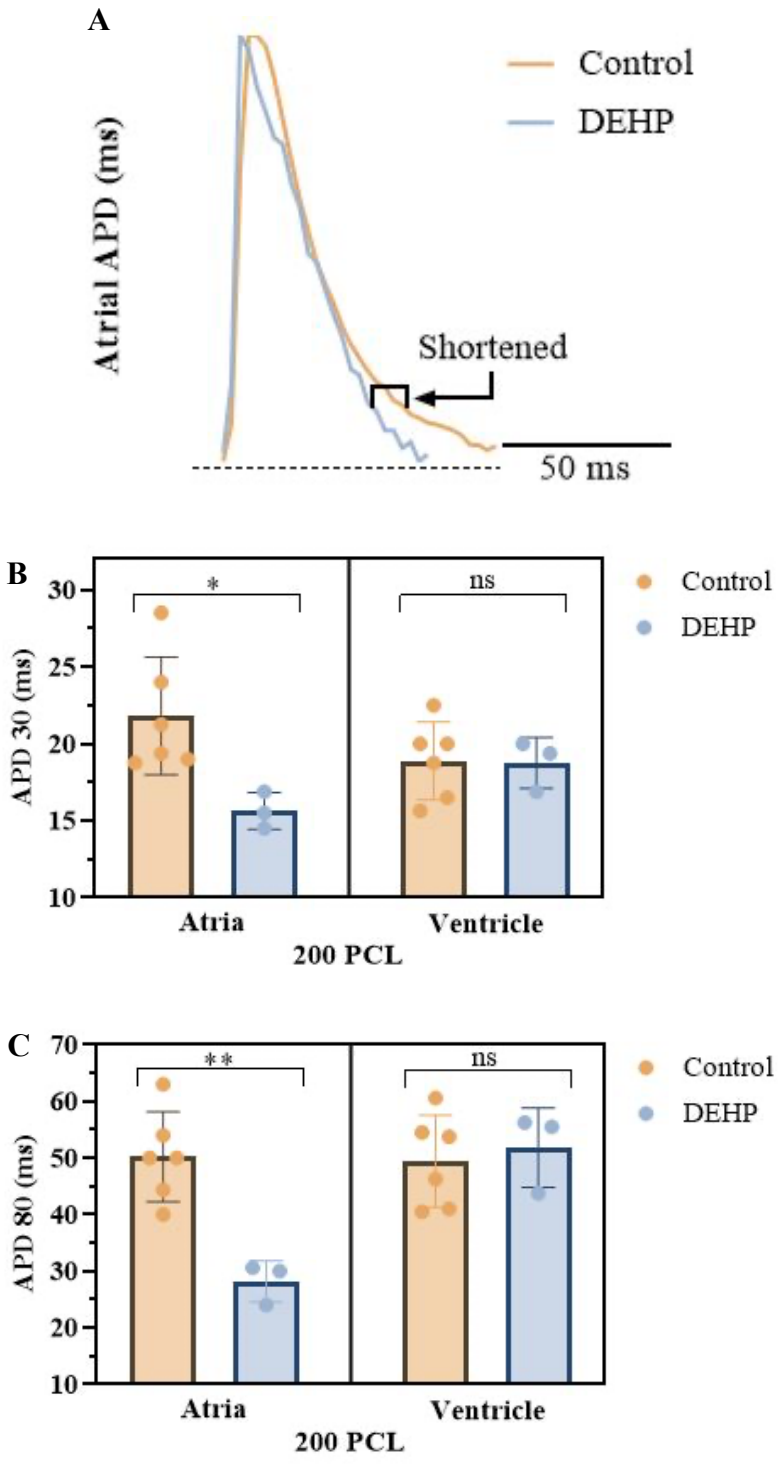

Figure 10: Action Potential Duration. **a** Atrial control and DEHP APD trace. **b** Average atrial action potential duration (APD) 30 at baseline and 60 minutes DEHP (*p<.05) vs. average ventricular APD 30 at baseline and 60 minutes DEHP. **c** Average atrial action potential duration (APD) 80 at baseline and 60 minutes DEHP (**p<.01) vs. average ventricular APD 80 at baseline and 60 minutes DEHP.

Chapter 5: Discussion

These studies were conducted to determine the direct effects of DEHP on cardiac function. Rubin et al. showed cardiac cell death in chick embryo heart cells from DEHP exposure.[58] While other studies showed significant impact on electrophysiology parameters, such as AVNERP lengthening, with exposure to MEHP.[27] The results from this study help improve understanding for how DEHP may impact cardiac function.

As hypothesized and proposed in the literature,[3] studies suggest DEHP acts in a similar manner as acetylcholine on the M2 receptor. Reduced cAMP levels result in slowed depolarization of the SA node, as they influence I_f current, generating a slowed heart rate.[26] Increased IK_{ACh} activity hyperpolarizes the action potential, resulting in a reduced slope of the action potential upstroke and contributing to a reduced heart rate.[19] When Langendorff perfused hearts were exposed to DEHP, the heart rate was greatly reduced. The sinus node recovery time (SNRT) was also significantly increased when exposed to DEHP, suggesting DEHP inhibits the ability of the sinus node to depolarize, resulting in a slowed recovery time.

ACh has been shown to contribute to cardiac vasoconstriction,[46] and the reduction in flow rate from DEHP exposure supports the hypothesis that phthalates may also interact with M2 receptors in coronary vessels. The observed steady decline in flow rate during control studies was consistent with a reduced oxygen demand and flow rate from the use of a mechanical uncoupler.[63] Current literature suggests mouse myocardial function to decrease about ten percent after 60 minutes of Langendorff perfusion.[37] The coronary flow rate in

control studies were reduced a comparable amount, therefore we cannot dismiss a modest decline in heart function during control perfusion.

AV block has been shown in MEHP exposed hearts[27] and PR segment/interval lengthening is representative of AV block.[32] These studies showed significant lengthening in PR segment/interval duration when exposed to DEHP, signifying first degree AV block.[23] In 78% of the studies, complete uncoupling between the P wave and QRS complex was observed, indicating a loss of communication between the atria and ventricles. This uncoupling also signifies third degree AV block or complete heart block.[69] Control studies showed no significant PR interval/segment lengthening or uncoupling between the P wave and QRS complex. This suggests DEHP induces AV block in exposed hearts.

Slowed conduction through the AV node was hypothesized if DEHP activates the M2 receptor[28] and M2 activation can slow electrical conduction through the AV node.[22] DEHP exposed hearts displayed the Wenckebach phenomenon at much slower rates than their baseline comparisons, showing significantly longer WBCL durations. Wenckebach phenomenon is a result of AV slowing, so these results indicate compromised AV conduction from DEHP exposure.[31] The AVNERP results indicate the ERP of the AV node increases with exposure to DEHP and DEHP reduced its ability to repolarize quickly. Control studies did not show significant lengthening in these parameters, so time alone does not appear to reduce conduction velocity. DEHP however, may lead slowed conduction through the AV node and a loss of gap junction communication or gap junction

uncoupling[11], indicated by WBCL phenomenon occurring at a slower PCL and ERP lengthening in the AV node.

An increased amount of IK_{ACh} activation should also be observed if DEHP acts on the M2 receptor.[28] Current literature states there are up to 5 times more M2 receptors in the atria than the ventricle,[24] so more IK_{ACh} activation should be observed in the atria than the ventricle. The reduction of APD 30 and APD 80 in the atria demonstrate this upregulation of IK_{ACh}. APD 30 and APD 80 showed no significant change in the ventricle. This supports the literature that states more M2 receptors are in the atria than the ventricle, and it also supports the hypothesis that DEHP stimulates this receptor in a similar manner to ACh. Further, it demonstrates a hastened repolarization and indicates its hyperpolarization could be contributing to a reduced heart rate with DEHP exposure.[41]

Chapter 6: Conclusion

Current literature suggests DEHP may impact cardiac function.[39,42,51] The results from the studies in this paper show direct cardiac effect from DEHP exposure. These studies suggest that DEHP does impact cardiac function by reducing automaticity, precipitating vasoconstriction of the coronary arteries, slowing AV conduction, and shortening atrial APD.

DEHP exposure resulted in a reduced heart rate and increased sinus node recovery, a similar effect that occurs with vagal stimulation[1]. DEHP exposure also resulted in coronary vasoconstriction, and previous studies show this with exposure to ACh[2]. Further, increased duration times in PR Interval, PR Segment, WBCL, and AVNERP parameters showed a significant delay in AV conduction when hearts were exposed to DEHP. Finally, AV block was noted in the majority of DEHP exposed hearts, which is another consequence of ACh stimulation.[28] ACh has been shown to delay conduction,[28] so these results suggest DEHP is activating the M2 receptor in a similar manner to ACh, as current literature has proposed.[3]

Future studies are necessary to fully understand the mechanistic effect of DEHP and will incorporate M2 antagonists to evade the effects of DEHP. Antagonists such as atropine have been shown to block effects of ACh,[36] such antagonists can be used to determine if the inhibitory effects of DEHP can also be blocked. The results of these inhibitory studies will further demonstrate if DEHP is acting on the M2 receptor. Secondly, a cAMP reporter will be used to analyze the amount of intracellular cAMP when exposed to DEHP. If these

levels are reduced, it would indicate DEHP is activating the M2 receptor and reducing the amount of intracellular cAMP.[64]

For over 40 years, the cardiotoxic effects of DEHP have been investigated[58] but the FDA still allows significant amounts of DEHP in medical devices.[51] The results from this study have shown DEHP to have a major effect on cardiac function, with reduced automaticity, coronary vasoconstriction, slowed conduction and shortened atrial APD. These results also suggest DEHP to act as a M2 receptor agonist because ACh activity results in similar cardiac depressive effects.[45] However, more mechanistic studies are needed to further understand how DEHP impacts cardiac function. DEHP exposure may have serious cardiac consequences and the impact DEHP has on exposed patients must be better understood.

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Appendix I

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