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Elucidating the Developmental Defects in Zebrafish Associated With the Cardiac Drug Verapamil

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ELUCIDATING THE DEVELOPMENTAL DEFECTS IN ZEBRAFISH ASSOCIATED WITH THE CARDIAC DRUG VERAPAMIL

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By

Blake Stephan Justis

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ABSTRACT

Birth defects are abnormalities in a developing organism that lead to a malformation in structure or function. Over half of birth defects have no determined cause; however, known causes occur by genetic anomalies, exposure to environmental agents (a.k.a. teratogens), or multifactorial reasons. To explain the unknown causes of birth defects, an area of focus in this study is to identify potential teratogens. Identifying these teratogens, is key to preventing future birth defects. An obvious source of teratogens in pregnant women would be that of pharmaceuticals. Thus, a main goal of this study is to identify drugs that cause birth defects. To ensure their cardiovascular health, some pregnant women need to take cardiac drugs for a variety of reasons. However, few studies have evaluated whether these drugs negatively affect developing embryos. This study uses the zebrafish model to assess the teratogenic effects of multiple cardiac inhibitors including verapamil, amlodipine, and 2,3-butanedione monoxime (BDM). The results of this study show that use of all three cardiac inhibitors impaired heart function at their lethal dose 50 (LD50). The severity and types of defects observed in non-cardiac tissues varied for each drug treatment. For example, verapamil exposed larvae exhibited yolk sac edema, a unique dorsal tail curvature, and altered jaw development. BDM-exposed larvae also exhibited yolk sac edema but had a different tail curvature and jaw defect distinguishable from verapamil treatment. Whereas, larvae exposed to amlodipine, which is in a similar class of drugs to verapamil, only exhibited a mild yolk sac edema and no defects in non-cardiac tissues. This study revealed that all the drugs tested impaired embryonic heart contraction, and led to cardiovascular defects, at their LD50. However, verapamil showed unique defects in non-cardiac tissues likely caused by the direct action of verapamil on these tissues, and not due to impaired blood flow. Based on similar defects observed in *pkd2* (*polycystin2*) mutants, I hypothesized that verapamil may inhibit Pkd2 (a non-selective cation channel) in the kidney, skeletal muscle/notochord, and pharyngeal cartilage, and thus providing a plausible explanation for the defects of yolk sac edema, dorsal tail curvature, and jaw formation. Results from this study identified concentrations and developmental defects that could negatively affect human embryos. More studies are needed to validate these findings in a mammalian model.

KEYWORDS: Verapamil, Amlodipine, 2,3-Butanedione monoxime, Teratogen, Zebrafish

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INTRODUCTION

Causes of Congenital Defects

Congenital defects (also known as, birth defects, congenital anomalies, or congenital abnormalities) are a very serious issue worldwide. In 2014, there was an estimated 3.2 million children born with severe birth defects (Kurdi and Majeed-Saidan, 2015). Of these 3.2 million children, an estimated 270,000 children died before 4 weeks of age due to these defects (Kurdi and Majeed-Saidan, 2015). Congenital defects result in health problems and/or physical abnormalities in normal structure or function that occur during gestation. These defects can be identified prenatally, at birth, or later in life (Kurdi and Majeed-Saidan, 2015). Some common examples of congenital defects include hemoglobin disorders, congenital heart defects, neural tube defects (anencephaly, spina bifida), reduction of the upper and lower limbs, and Down syndrome (Christianson et al., 2006). In addition to health concerns that manifest at or after birth, congenital defects account for about 10% of fetal deaths during gestation in 2014 in the U.S. (Hoyert and Gregory, 2014). Even in those individuals whose defect is not neonatally lethal, it will likely cause issues over the course of a person's life. For example, the average life expectancy of someone with Down syndrome is 60, about 18 years less than the national average (Esbensen, 2010).

There are many possible causes of congenital defects. New or acquired genetic anomalies (mutations in a gene or chromosomal abnormalities) in the developing organism can be a cause of these defects. For example, a mutation in *Fibroblast Growth Factor Receptor 3* (*FGFR3*) causes achondroplasia or dwarfism (Prescott and Wilkie, 2007). Due to this mutation, *FGFR3* becomes over-activated, which leads to suppression of chondrocyte growth and maturation. This

in turn leads to lower growth plate size and perturbed bone elongation (Prescott and Wilkie, 2007). Different mutations in *FGFR2* lead to craniosynostosis type disorders (defects in skeletal bone fusion) like Crouzon's, Apert's, Pfeiffer's and Beare-Stevenson syndromes (Prescott and Wilkie, 2007). It is also noteworthy to add that mutations in some genes may cause multiple defects in the body due to the pleiotropic role of many genes. Hence, multiple congenital defects may arise as is the case with mutations in the *c-KIT* gene that causes piebaldism, anemia, and sterility (Fleischman, 1992). Other congenital defects may arise due to the combination of alleles in different genes in an embryo. Because the combination of multiple mutations in genes/alleles can be the cause of a congenital defect, this makes linkage analysis very difficult to perform (especially with limited family history); thus, identification of the genetic cause may be very difficult to determine. In addition to defects in genes, some congenital defects are caused by chromosomal abnormalities such as a duplicated, deleted, or translocated regions of a chromosome, or in some instances too many or too few chromosomes. An example of a chromosomal abnormality would be trisomy of the 21st chromosome, which causes Down syndrome—a conditioned marked by characteristic facial features, intellectual disability, and in some cases congenital heart defects (Kazemi et al., 2016).

There are many genetic anomalies that cause congenital defects, some that are known and others still requiring discovery due to several reasons (combination of multiple mutant alleles causing a birth defect, rare genetic diseases that are not well studied, new/germline mutations preventing studying the family history, identification of mutations not yet linked to a particular birth defect, etc.). However, it has become increasingly challenging to determine the cause of a congenital defect because environmental factors are known to influence embryonic/fetal development. It is thought that exposure to environmental factors has significant influence on the

formation of birth defects, as 10% of birth defects have a cause directly linked to the environmental agent (Brent, 2004). Further, 50% of birth defects have no known cause (Lobo and Zhaurova, 2008); thus, a percentage of these defects may be due to exposure to an environmental agent. A specific name for these environmental factors is teratogens. Teratogens are external agents that cause a developmental abnormality after exposure to a developing embryo/fetus (Alliance and Health, 2008). There are different categories of teratogens including infectious diseases (viral and bacterial), parasites, environmental chemicals, drugs, and physical agents (temperature, radiation or mechanical forces) (Alliance and Health, 2008). Pregnant women can be exposed to these different factors in a variety of ways including drinking contaminated water, eating contaminated foods, taking medications, and acquiring infectious diseases. Infections can cause issues for developing organisms. For example, infection of the mother by the Zika virus during pregnancy causes the virus to reach the developing embryo/fetus, and this infection causes the brain and skull of the baby to develop much smaller than normal, a condition known as microcephaly. Laboratory studies have determined the cause of the Zika-induced microcephaly is an impairment to neurogenesis, or new neuron formation (Rosa-Fernandes et al., 2019). In another category, exposure to chemicals can also cause congenital abnormalities. An example of this is the use of Agent Orange, a tactical herbicide used during the Vietnam War. Specifically, 2,3,7,8-Tetrachlorodibenzodioxin (an active component of Agent Orange) has been shown to cause a variety of deformations in animal models including kidney malformations, cleft palates, and organ/tissue edema (NIOSH, 1984). A meta-analysis of epidemiological studies looking at exposure to 2,3,7,8-Tetrachlorodibenzodioxin and occurrence of birth defects found that people exposed to this agent had about double the chance of having offspring with some sort of congenital defect (Ngo et al.,

2006). An infamous example of a medication causing developmental defects is the case of thalidomide. This drug was prescribed to pregnant women to treat morning sickness. During its use in pregnant women, over 10,000 children were born with debilitating deformities that included limb, eye, ear, face, spinal, and internal organ abnormalities (Vargesson, 2015). Regardless of what kind of teratogen a developing organism is exposed to, these teratogens will ultimately target specific biochemical pathways, alter developmental gene expression, or mutate developmental genes in embryonic/fetal tissues resulting in a change in normal development.

A more elusive teratogen, that is much less reported, is that of mechanical force. It is highly evident that mechanical forces are essential aspects regulating development of tissue organ size, morphogenesis (i.e. tissue shape), and stem cell biology (Mammoto and Ingber, 2010; Vining and Mooney, 2017). For example, stretching or compression of developing organs, such as the developing wings in fruit flies, have been hypothesized to be one way to control organ size during development (Aegerter-Wilmsen et al., 2007). Mechanical forces are also important for craniofacial development. A combination of pressure forces for cartilage development, shearing forces for bone development and dilation forces in muscle development all contribute to normal craniofacial development (Radlanski and Renz, 2006). Another example of mechanical forces in development is the need for the correct level of fluid pressure in the developing lung in order to obtain correct development (Hogan, 2018). Without these correct fluid pressures, there will be malformations in the lungs that will result in lifelong abnormalities. Developing tissues can sense and respond to different kinds of forces from tissue tension forces, to mechanical stress and strain, to compression (Hernández-Hernández et al., 2014). The effect of mechanical signals on regulating development is an emerging field, and hence little is known on how perturbations to

mechanical force results in birth defects. Exceptions are studies exploring how perturbations in mechanical forces cause defects in the developing cardiovascular system.

The Role of Mechanical Forces in Regulating Development of the Cardiovascular System

The most informative studies on the role of mechanical force in regulating development have focused on the role of blood flow forces (hemodynamic force) on the developing heart and vasculature. Studies in many model organisms have shown that altering hemodynamic forces during development lead to abnormal development of the heart. For example, blocking the vitelline vein in chick embryos obstructs blood flow, lowering hemodynamic load. This leads to a variety of malformations, including changes in endothelial cell arrangement, stiffness in the ventricular wall, ventricular septal abnormalities, semilunar valve anomalies, and malformations in the pharyngeal arch artery (Midgett and Rugonyi, 2014). Moreover, in zebrafish, surgical procedures that lower cardiac output have been shown to produce heart defects including third heart chamber abnormalities, looping issues, and impaired valve formation (Lindsey et al., 2014).

In the mouse, hemodynamic force has been shown to be important in vascular remodeling, normal aortic arch formation, and normal placental development (Culver and Dickinson, 2010). Elucidating the role of hemodynamic forces in cardiovascular development in mice is at the heart of the research in the Udan lab. The Udan lab has determined that hemodynamic force is responsible for regulating vessel hierarchies (Udan et al., 2013), proper morphogenesis of the heart (Hoog et al., 2018), and maturation of the vasculature (Padget et al., 2019). Considering that hemodynamic force is required for normal development of the cardiovascular system, then disrupting blood flow could be an additional cause of a congenital

heart or vascular defect. However, an important question remains. What could cause impairments in blood flow to occur in the first place?

Elucidating the Potential Causes to Impaired Blood Flow During Embryonic Development

There are two possible causes of impaired blood flow during embryonic development. First, it is possible that an embryo may have a mutation in a gene that is required for heart contraction. For example, several mutants have been made, across different model organisms, disrupting an array of genes required for myocardial function. Mutations in (or morphants of) *troponin T type 2a* (*tnnt2a*, zebrafish) (Liu et al., 2019), *Sodium-calcium exchanger 1* (*Ncx1*, mouse) (Conway et al., 2003), *Myosin light chain 7* (*Myl7/Mlc2a*, mouse) (Huang et al., 2003) have been generated. Though people can inherit hypomorphic mutations in these genes, these do not significantly impair heart contraction, as patients are likely heterozygous for the mutation, and have one functional copy, allowing hearts to contract normally, and for the embryo to develop to adulthood. Albeit, other mutations may be semi-dominant, as the mutated copy may not initially have affects in the embryo, fetus or child, but manifestations may result in familial cardiomyopathies and fibrillations (Schwartz et al., 1995). The only way mutations in these genes will affect embryonic blood flow is when both copies of the genes are mutated. However, I speculate that in this instance, hearts would fail to contract resulting in embryonic lethality, as blood flow is also required to support oxygenation, nutrient supply, and waste removal.

A more reasonable hypothesis, explaining why some people are born with congenital heart defects, is that there may have been a temporary impairment in heart contraction during embryogenesis. This would likely be caused by a transient exposure to a chemical substance that may impair heart contraction. Therefore, identification of teratogens that impair embryonic heart

contraction may help to elucidate the potential causes of congenital heart and vascular defects. An obvious way in which human embryos/fetuses may be exposed to a substance that impairs heart contraction would be if a pregnant mother takes a heart medication to treat hypertension or other cardiac concerns. These concerns are common in pregnant women, and must be treated, as there are many changes that occur to the cardiovascular system that may cause a cardiovascular issue or augment one that is already present (Halpern et al., 2019). For example, marked elevations in estrogen levels that occurs during pregnancy can activate the Renin-Angiotensin-Aldosterone system (described in more detail later), resulting in an increase in blood plasma volume (Halpern et al., 2019). Thus, heart medications may be needed by some women. However, not much is known about whether heart medications that are taken by pregnant mothers can reach the developing embryo, and if the drug does reach the embryo, little is known about the drug concentrations that will impair embryonic heart function, and thus impair heart development.

In addition to the potential effects of cardiac drugs on impeding cardiac function of the embryo/fetus, it is also unknown whether these drugs have potential effects on non-cardiac tissues. If embryonic/fetal exposure results in developmental defects in non-cardiac tissues, then it will be important to identify whether these defects are caused by impaired blood flow (due to impaired heart contraction), or if the drugs act directly on these non-cardiac tissues. Currently, very few studies have addressed these concerns, requiring the scientific community to follow through on these studies, by using model organisms.

Zebrafish as a Model System to Identify Teratogen Effects of Cardiac Drugs

The use of animal models has been very successful in helping to identify teratogens. One organism that has been very useful is the zebrafish model. There are many benefits with using zebrafish including relative ease of care, production of 50-300 eggs at a time, external fertilization (with a functional heart forming by 2 days post fertilization (dpf), and the transparency of developing embryos (Burke, 2016). Another useful feature is the idea that zebrafish share 70% of genes with humans and have many conserved biochemical pathways (Burke, 2016), and developmental processes are relatively conserved between different vertebrate taxa; thus, information gathered from zebrafish studies could be applicable to humans (Nishimura et al., 2016). Zebrafish embryos have been the gold standard for large-scale screening of chemicals/drugs to determine both the teratogenic and toxic effects of potential teratogenic chemicals/drugs, and to determine potentially harmful concentrations (Ali et al., 2014; Yamashita et al., 2014). However, few studies have been conducted that provide a thorough analysis of the teratogenic effects of cardiac drugs. This is the premise of this thesis study.

Cardiac Drugs

A cardiac/heart drug is a medication that causes a physiological change in the heart and associated vessels. There are many types of heart medications with common ones being angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, myosin inhibitors, and calcium channel blockers. While all these medications have similar end goals, they work in different ways.

Angiotensin II is an important molecule in the renin-angiotensin system (RAS) (Piepho, 2000). RAS is important for many physiological functions including maintaining blood pressure. When angiotensin II is activated, it binds to angiotensin type I receptor, which then causes a multitude of downstream events including vasoconstriction, and sodium and fluid retention that leads to hypertension (Piepho, 2000). ACE inhibitors work by neutralizing the ability of the ACE complex to convert the biologically inactive Angiotensin I to the active Angiotensin II, thus mitigating the negative effects brought about by Angiotensin II (Piepho, 2000). There are currently 10 ACE inhibitors available in the United States ("Angiotensin-Converting Enzyme Inhibitor (ACE inhibitor) Drugs | FDA," 2015). ACE inhibitors help with a wide range of medical conditions including hypertension, congestive heart failure, and use after heart attacks to increase survivorship (Herman et al., 2020).

Beta-blockers are another important class of cardiovascular drugs. There are different types of beta-blockers. Non-cardiac specific beta-blockers indiscriminately block the β1, β2, and β3 adrenergic receptors (Farzam and Jan, 2019). Therefore, this would illicit a similar effect in the heart, but may have other physiological effects in other areas of the body. Cardiac specific beta-blockers target the β1 receptor, specifically in the heart (Farzam and Jan, 2019). β1 specific beta-blockers work by acting as a competitive antagonist to β1 receptors which prevents the ability of epinephrine and norepinephrine to bind to β1 receptors in the myocardium of the heart (Farzam and Jan, 2019). Beta-blockers affect sinoatrial and atrioventricular node conduction, and by diminishing β1 receptor activity leads to reduced heart rate and reduced cardiac contractility (Man et al., 2010). The binding of hormones to β1 receptor also leads to the release of the renin enzyme, which is important in the RAS system, and leads to an increase in blood pressure (Farzam and Jan, 2019). When beta-blockers are introduced, there is a combined function of

lowering cardiac output and blocking the release of renin, and this leads to a decrease in blood pressure (Farzam and Jan, 2019). There are many FDA approved beta-blockers including atenolol, bisoprolol, and metoprolol that are cardiac specific beta-blockers (Saseen, 2012). Beta-blockers have been FDA approved for use in tachycardia, heart arrhythmias, hypertension, and other conditions (Farzam and Jan, 2019).

Myosin inhibitors are a class of chemicals that inhibit skeletal, smooth muscle, and/or cardiac myosin. The myosin inhibitor used in this thesis project is 2,3-butanedione monoxime (BDM). BDM is a well-studied chemical that has been shown to diminish cardiac contraction and output (Watanabe et al., 2001). It is hypothesized to work by inhibiting the ATPase of myosin 2 (Hall and Hausenloy, 2016). This myosin inhibition also effects the release of calcium which causes a variety of events leading to inotropic (lowered heart muscle function) results (Gwathmey et al., 1991). BDM was used in this study to compare to verapamil, which is in another class of drugs and has a different mechanism of action but results in a similarly low cardiac output state.

Calcium channel blockers are a class of drugs that work by blocking the action of L-type calcium channels (also known as dihydropyridine receptors) of cardiac, smooth, and skeletal muscle (Taddei and Bruno, 2018). There are different types of calcium channel blockers and they are grouped into different classes based on their affinity for certain tissues and their chemical structure (Taddei and Bruno, 2018). There are three main classes of calcium channel blockers: benzothiazepines; phenylalkylamines; and dihydropyridines (McDonagh et al., 2005). The dihydropyridines include the drugs amlodipine, bepridil, felodipine, isradipine, nicardipine, nifedipine, and nisoldipine (McDonagh et al., 2005). Dihydropyridines have a higher specificity towards vascular smooth muscle, and therefore their antihypertensive properties are attributed

more to vasodilation rather than inotropic effects on the heart (McDonagh et al., 2005). The non-dihydropyridines calcium channel blockers, benzothiazepines (diltiazem) and phenylalkylamines (verapamil), tend to have less affinity to vascular smooth muscle and instead have a direct effect on the myocardium (McDonagh et al., 2005). These direct effects of the myocardium lead to the depression of the sinoatrial (SA) and atrioventricular (AV) nodes (McDonagh et al., 2005). Therefore, the benefits seen from use of non-dihydropyridine calcium channel blockers are due to inotropic effects rather than vasodilation (McDonagh et al., 2005). In general, calcium channel blockers can be prescribed for a variety of ailments including hypertension, angina, tachycardia, and atrial fibrillations (Godfraind, 2017).

The Potential Teratogenic Effects of Cardiac Drugs

Around 2 percent of pregnancies are complicated by heart disease, and of those 2 percent, about one third of the patients take heart medications during pregnancy (Halpern et al., 2019). Cardiac drugs may be of concern during pregnancy due to risk of embryonic/fetal exposure. However, the use of cardiac drugs during pregnancy is warranted in many situations. For example, pregnant women may take beta-blockers to treat heart failure, or take nifedipine (a commonly used calcium channel blocker) to treat hypertension (Halpern et al., 2019). Verapamil specifically has important use during pregnancy as a treatment for supraventricular tachycardias, atrial fibrillations, and atrial flutters (Halpern et al., 2019). Thus, these medications need to be taken to ensure health of the pregnant mother.

Whether maternal exposure to these heart medications causes congenital heart defects in the embryo/fetus is difficult to elucidate based on the available evidence. There is missing information due to a lack of comprehensive data sets in animal studies. For example, studies

involving the exposure of nifedipine to pregnant rats were mostly performed at late pregnancy stages, and mostly focused on the effects of drug exposure on the birthing process (Abel and Hollingsworth, 1986; Gallagher and Gautieri, 1992; Tracy and Black, 1992).

Data from human studies are also limited, but trends show that maternal exposure to cardiac drugs may cause negative affects to the fetus or embryo. For example, in 21 pregnancies affected by pregnancy-induced hypertension, maternal treatment with nifedipine altered umbilical artery flow (measured by Doppler ultrasound) and affected fetal behaviors (Gazzolo et al., 1998). In a study assessing the first trimester, antihypertensive medications such as ACE inhibitors, have been shown to not result in malformations, but did increase the amount of miscarriages that occurred (Moretti et al., 2012). Though the authors concluded that ACE inhibitors are not teratogens, whether the increased rate of miscarriage was caused by a failure for the embryonic heart to function could not be determined since the subsequent death of the embryo was not assessed. Epidemiological studies have indicated that taking anti-hypertensive medications during pregnancy increases the risk of birth defects (Caton et al., 2009; Podymow and August, 2008). Based on results from these studies, more research in animal models needs to be done during embryonic periods of development as the early embryonic heart forms and matures to determine the effects of various cardiac medications.

The drug of focus in my thesis study was verapamil hydrochloride. Verapamil is a phenylalkylamines class calcium channel blocker, thought to be specific to L-type calcium channels (Wang et al., 2017). The primary mode of action involves cardiac L-type calcium suppression of the SA and AV nodes in the heart (McDonagh et al., 2005). Results from the literature provides evidence that it has some effect on vascular smooth muscle calcium channels as well, but its main physiological effect is the direct inotropic effect on the heart (Wang et al.,

2017). Verapamil was first synthesized by the German pharmaceutical firm Knoll when attempting to make a synthetic analog of the poppy constituent papaverine (Davies and Hollman, 2002). In 1963 the scientist Albrecht Fleckenstein was the first to study the calcium blocking ability of verapamil (Davies and Hollman, 2002). More research was done on the applications of verapamil, and it was first approved for use in the United States in 1981 (*Verapamil*, 2012). It is currently approved for treatment in hypertension, angina, supraventricular tachycardia, and other arrhythmias (Fahie and Cassagnol, 2019).

The information on the fetal safety with verapamil is limited (Halpern et al., 2019). Its current FDA classification is category C ("Verapamil," n.d.). Category C means that there have been previous studies that show some sort of adverse effect on the embryo, and there are no well controlled human studies, but the drug may still be used if it is needed ("FDA Pregnancy Categories - CHEMM," 2008). The lowest level of the FDA classification of drugs during pregnancy is category X. Category X means that studies in humans or animals have demonstrated fetal abnormalities ("FDA Pregnancy Categories - CHEMM," 2008). While previous studies may have shown adverse effects of verapamil, what my study is investigating is whether verapamil causes fetal abnormalities mentioned in the Category X classification. In addition to fetal exposure to verapamil through maternal use of pharmaceuticals, verapamil is commonly found in water sources due to contamination (Patel et al., 2019). Concentrations of up to 7340 ng/L have been reported in bodies of water receiving run-off from pharmaceutical plants (Patel et al., 2019).

Few studies have assessed the teratogenic effects of verapamil in zebrafish; however, since these studies were performed as large-scale screens assessing a host of many other chemicals/drugs, very little information on verapamil exposure has been collected (Ali et al.,

2014; Yamashita et al., 2014). The teratogenic effects of verapamil that have been observed include lowered heart rate, edema of the pericardial sac (sac around heart), changes in heart size, uninflated swim bladders, and jaw deformities. A majority of these defects mimic expected results upon embryonic exposure to cardiac drugs, such as the lowered heart function and other phenotypes typically associated with decreased blood flow including pericardial edema, uninflated swim bladder, and changes in heart morphology. However, deformity in the jaw is of interest due to it being an unexpected non-cardiac abnormality.

Hypothesis

Since the non-cardiac defects associated with verapamil could be from lowered cardiac function, I will test the hypothesis that verapamil directly impairs embryonic cardiac function and development of non-cardiac tissues like the jaw via different mechanisms. To determine whether jaw deformities were a common defect associated with decreased blood flow or a phenotype caused by the direct actions of verapamil on cartilage development, a goal of my thesis was to compare and contrast the teratogenic effects of verapamil exposure to exposure with other cardiac drugs such as amlodipine besylate (dihydropyridine class of L-type calcium channel blocker) and 2,3-butanedione monoxime (a myosin inhibitor). Another goal was to more thoroughly characterize and quantify the teratogenic effects associated with verapamil exposure as there could be other effects that were not observed in the initial large-scale screens. As a final goal, I sought to determine the lethal dose 50% (LD50) at which these drugs impair development/health of the embryos/larvae and at what concentration they impair heart function/development. Results from my study will provide valuable information on the

concentrations of these drugs that may impair human development and determine which medications are potentially safer for use by pregnant mothers.

METHODS

Zebrafish Care

Zebrafish (*Danio rerio*) of the wildtype Tübingen (TU) line were used in this study. They were housed in two different ways. In the initial system, two 20 L tanks were used to house the fish. With a maximum amount of fish per tank at 45. These tanks had power filters to remove organic matter and a biological filter to facilitate the growth of beneficial nitrifying bacteria. In the latest system, the fish were housed in an aquatic habitat (AHAB) system. This system allowed for housing of nine 10 L tanks and three 5 L tanks organized on a rack, as well as a 30 L reservoir/basin that acts as a biofilter, and 10 L worth of tubing/filtration, resulting in a total of 145 L of system water. In the AHAB, water continuously cycled throughout the system. The water started in a basin at the bottom of the AHAB where lava rocks and bio-balls were kept to retain nitrifying bacteria. The water was then pumped into a sock filter (to eliminate particulate matter), flowed through a UV filter (to kill bacteria and fungi), flowed to the top tanks housing the fish, and finally flowed over the top of these tanks into a collection system and back down into the basin.

In both housing situations, male and female zebrafish were cohoused together, and were exposed to a 12-hour light and 12-hour dark cycle. They were given a set amount of food per day in order to ensure overfeeding did not occur. Zebrafish were fed Zeigler zebrafish diet food (Zeigler #388765-101-686). In order to make sure the water was at the correct pH, ammonia, nitrate, and nitrite levels for the fish, water quality checks were done daily. Various kits were used to test the water quality, these include API Ammonia (API # LR8600), API Nitrate (API #

LR1800), API Nitrite (API # 26) and pH strips (Ricca Chemical Company # 8882-1). Also, water change outs were done daily. Change outs were done using new system water. System water was comprised of either ultrapurified distilled water (for the initial two 20 L tanks), or more recently for the AHAB system, tap water that had undergone reverse osmosis (R.O). In both cases, salts (Instant Ocean, #SS15-10) were added back to maintain an optimum salinity needed for zebrafish (0.25 PPT). The amount of new system water added varied depending on the water quality levels. Ammonia levels higher than 0.25 PPM, nitrate levels higher than 5 PPM, and/or nitrite levels higher than 0.25 PPM would require up to a 30% daily change out; however, when levels were lower than these values, daily change outs as low as 7% were performed. In some cases, the amount of ammonia build-up was so high that the water change outs were insufficient. In this situation, ammonia remover (Seachem # B00025694O) was added, as per the company's recommended dosage. In addition, to promote colonization of the nitrifying bacteria, bacterial supplements (Imagitarium # 728484) were administered as needed. In cases when pH was too low, the system water was supplemented with a 10% solution of sodium bicarbonate in system water (Fisher #S233-500), and the correct amount was added until the water returned to a neutral pH. In cases when the pH was too high, a larger change out of system water was done. All zebrafish were handled in accordance with our Missouri State University IACUC approved protocol (18.003.0).

Zebrafish Spawning

Spawning was done in two ways, the first being the use of in-tank spawning trays. Just after the lights were turned on in the morning, the in-tank spawning trays were placed in the tanks (suctioned onto the side of the tank) and left in the tanks for a few hours. The rationale for

why spawning trays stimulate the mating behavior is that the trays create a shore-like surface and have a grating system, which provides a safe environment for the fertilized eggs to collect. Within hours, zebrafish mate over the top of spawning trays, and as the eggs become fertilized, they fall through the plastic grates, and collect into the tray, preventing the fertilized eggs from being eaten by the zebrafish.

The second way embryos were collected was using an isolation tank. In this approach, two males and one female were captured via net and put into an isolation breeding tank in the morning. The fish were then left in the breeding tank undisturbed for a few hours. This tank has a grate separating the top from the bottom. The zebrafish were in the top, and when they mated, the fertilized embryos floated down through the grate and resided safely at the bottom for collection. In both approaches, embryos were collected from the tray/tank, and selected/washed to ensure that the embryos were developing properly and had few contaminants (bacteria/fungi).

Embryo Selection and Washing

After completion of mating, only viable embryos were selected for experiments. This helped to ensure that any lethality observed in experiments was caused by drug exposure, and not due to the usage of nonviable embryos. To screen for viability, the embryos were observed under a dissecting microscope, and they were sorted based on whether the animal pole of each embryo exhibited cell divisions as this would be an indication that fertilization has happened and that the embryo has underwent cleavage. Of the cleavage stage embryos, those that were 0.75 - 2.25 hours post fertilization (hpf) (or 4-128 cell stage) were selected for experiments; whereas, embryos that exhibited a visible, structural issue were discarded. Examples of structural issues include a torn chorion, asymmetry in the embryo, or what appeared to be multiple different areas

of development in the animal pole. To clear debris from the embryos, the embryos were moved to a 60 mm petri dish (MidSci #667665, city) and pre-washed by carefully pipetting up and down to remove any debris from the outside of the chorion. The embryos were then pipetted up and dispensed into a 60 mm petri dish within a sterile cell strainer (Fisher #22-363-547) and submerged in egg water. Egg water is made using 1 L of RO water with 1.5 ml sodium bicarbonate stock (100 g sodium bicarbonate in 1L RO water) and 1.5 ml of instant ocean stock salts (4 g instant ocean in 100 ml of RO water).

In order to reduce the potential for contamination in our experiments, all the embryos were bleach washed. The protocol for bleach washing was to place the embryos in the cell strainer and submerge the strainer in a 0.00189% bleach (sodium hypochlorite) solution for 5 minutes. Directly following this initial wash, the embryos would undergo 3 egg water washes lasting 3 minutes each. In these washes, the embryos were moved in the strainer from one dish to another. These embryos were then able to be used in the experiments.

Experimental Set-up

Since the working concentrations used in these experiments were small, solutions needed to be made with dilutions. To do this, an initial stock solution was made with a chemical/drug and then this was carefully diluted to the proper working concentration. Twenty-four well plates were used to house the embryos for the experiments (Midsci #24WHARVEST). Solutions were made and then placed into the different wells, and each well (1-24) was documented so that drug types/concentrations and untreated controls (egg water alone) were recorded. Then, two embryos were placed in each well. The embryos were then observed over a period of 5 days with imaging on days 4 and 5. For imaging, an Amscope dissecting microscope (Amscope #SM-1TSX-L6W)

was used with an attached 14MP Amscope camera (Amscope #MU1400). After imaging, embryos were euthanized via MS-222 (Syndel # TRIC-M-GR-0010) overdose in accordance to our IACUC approved protocol (IACUC: 18-003.0).

Chemicals

Verapamil was acquired from Sigma-Aldrich (V4629-1G Lot# MKBV4993V). 2,3 butanedione monoxime (BDM) was also from Sigma-Aldrich (B0753-25G Batch # 0000025534). Amlodipine besylate was acquired from Tokyo Chemical Industry (A2353 Lot# ZFR8F-BH). All chemicals were stored in a solid state, and fresh stock solutions were made every experiment. For all experiments egg water was used as a solvent.

Determining the LD50 Concentrations

When a chemical is first used, the LD50 first needs to be established. The LD50 is the concentration of the drug/chemical at which 50% of the animal model (embryos/larvae) die at that concentration. The LD50 was chosen for the experiments, as this is the point at which some embryos/larvae would be affected by the drug and some would not, and it is commonly used procedure in Zebrafish teratogen screening when testing an effective concentration that causes phenotypic changes (Ali et al., 2011). A standard range of concentrations was first used to get an estimate of the LD50. The standard range consisted of 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml concentrations. The results of this preliminary screening directed future experiments to find the LD50 for each drug/chemical, this method was used for amlodipine and verapamil. Alternatively, in the case of BDM, the chemical had been used before in experiments involving zebrafish and the previously documented concentration was used as a starting point. For the tests

at or around the presumed LD50, at least 3 independent experiments with 8 technical replicates were done in order to ensure the accuracy of the LD50 concentrations.

For my experiments, the LD50 was determined by assessing death of embryos 4 dpf, which was also the point in which embryos then become larvae. Some embryos did not make it past the early stages of development, so death was observed by the presence of an unhatched embryo with apoptotic tissue that appeared as a white color. Other embryos developed further, and death was assessed by either decomposition of the larvae or complete cessation of heart contraction along with lack of touch response. After the LD50 was established, further analysis of embryos at this concentration was done to characterize morphological changes

Imaging the Larvae and Morphometric Analysis

Two pictures of the embryos were taken at 40X total magnification, one photo of the anterior and one photo of the posterior. One photo of each embryo was taken at 15X total magnification as all of the embryo could be captured in one photo at this magnification. Photos were oriented with the anterior of the larva to the left and the dorsal side of the larva oriented upward.

Morphometric analysis was done on the 4 and 5 dpf larvae, looking at various phenotypes of interest (pericardial edema, abdominal edema, tail curvature), and measurements were made using the ImageJ program (https://imagej.nih.gov/ij/index.html). Pericardial edema and yolk sac edema analysis were done using the line tool. Distance was pre-calibrated using known distances on the same magnification that the image was taken. To assess pericardial sac edema, measurements were taken by drawing lines perpendicular to the body axis from the area of the

heart to the edge of the sac. This line was then analyzed by ImageJ and the length was recorded. Two lines were drawn and measured, and the average was used for statistical analysis.

For the yolk sac edema measurements, lines were drawn perpendicular to the body axis from the posterior side of the area of the swim bladder down to the edge of the yolk sac. This line was then analyzed by the measurement tool on ImageJ and the length was recorded. Two lines were drawn per embryo and the average of the lines was used in the statistical analysis.

The tail curvature analysis technique was adopted from a technique used to measure bird claw curvature (Birn-Jeffery et al., 2012). Using the angle tool in ImageJ, an angle was drawn from the dorsal side of the zebrafish, to the middle of the dorsal side of the tail, and then finally to the tip of the tail. Deviations from 180 degrees would then represent curvature in the tail. Knowing this value, the angle measurement was subtracted from 180 to obtain the final amount of deviation.

Statistical analysis was done in Excel. For each group (chemical at either 4 dpf or 5dpf), an F-test was initially done to compare the variance to the controls. Once this was determined, a 2-sample T-test was performed to look for significance between the experimental and control groups.

Cartilage Staining

A no-acid cartilage stain was used for staining of the embryonic cartilage in the zebrafish at 4dpf. First, the larvae were euthanized in accordance with our IACUC approved protocol (18.003.0). Then the larvae and 1 ml of the euthanizing solution (4 mg ms-222, ~1 ml of 1M tris [pH 8], 9 ml water) was transferred into a 2 ml round-bottom microcentrifuge tube (Eppendorf #022363352). The larvae were fixed by adding 1 ml of 4% paraformaldehyde in phosphate-

buffered saline (PBS) and nutated at room temperature for 50 minutes. Then, the solution was removed, and the larvae were rinsed two times quickly with 1.5 ml of PBS. The larvae were then rinsed one time with 1.5 ml of 50% ethanol (Decon Laboratories #64-17-5). The first 50% ethanol wash solution was removed, and fresh 1.5 ml of 50% ethanol was added to the tubes, and the tubes were nutated at room temperature for 10 minutes. The 50% ethanol was removed and a quick wash with 1.5 ml of 0.04% Alcian Blue (Newcomer Supply #1002A), 10 mM magnesium chloride ($MgCl_2$) (Sigma #M8266) in 80% ethanol was done. Then, the previous solution was removed and a fresh solution of 1.5 ml of 0.04% Alcian Blue, 10 mM $MgCl_2$ in 80% ethanol solution was added. This was nutated at room temperature overnight.

The next day the last solution was removed and a quick rinse with 1.5 ml of 80% ethanol, 10 mM $MgCl_2$ solution in water was done. This was then removed and a fresh 1.5 ml of 80% ethanol, 10 mM $MgCl_2$ solution was added, this was then nutated at room temp for 5 minutes. A quick rinse with 1.5 ml of 25% ethanol was done, then the solution was replaced with fresh 1.5 ml of 25% ethanol and this was nutated at room temperature for 5 minutes. This solution was removed and a quick wash with 1.5 ml of a 30% hydrogen peroxide (Fisher #7722-84-1) and 20% potassium hydroxide (KOH; Fisher # 1310-58-3) solution was done. This was then removed, and 1.5 ml of the same solution was added back and allowed to sit at room temperature with the cap open for ten minutes. The bleach solution was removed, and a quick wash was done with 1.5 ml of a 25% glycerol (Fisher # G334), 0.1% KOH solution in water. This was then removed, and a fresh 1.5 ml of 25% glycerol, 0.1% KOH solution in water was added back and nutated at room temp for 15 min. This solution was removed and a quick wash with 1.5 ml of a 50% glycerol, 1% KOH solution in water was done. The solution was replaced with a fresh 1.5

ml of 50% glycerol, 1% KOH solution and was nutated at room temperature overnight. The next day, a trypsin digestion was performed.

The second portion of this stain was to do a trypsin digestion to clear the tissues. A saturated (4.7%) solution of sodium tetraborate (Fisher #1303-96-4) was made in PBS. Hydrochloric acid was then added to this solution until the pH was 7. This was then diluted down to the working concentration of sodium tetraborate (3.5 ml of saturation sodium tetraborate and 6.5 ml of PBS). Five mg of trypsin (Sigma #T1426-100MG) was then added to the diluted solution of sodium tetraborate, this was then the working solution for the trypsin digestion protocol. The 50% glycerol, 1% KOH solution was removed from the larvae, and 1.5 ml of PBS was added. The larvae were soaked in the PBS for 3 minutes. The PBS was removed, and 1.5 ml of the working trypsin digestion solution was added, and the larvae sat at room temperature for 2.5 hours. The trypsin solution was then removed and 1.5 ml of 30% hydrogen peroxide, 20% KOH solution was added, and the larvae sat at room temperature for 2 hours with the lid of the tube open. This solution was then removed, and imaging was performed similarly as described previously.

RESULTS

Determining the LD50 of Each Cardiac Drug Upon Exposure to Zebrafish Embryos/Larvae

To properly assess the teratogenic actions of various cardiac drugs on zebrafish embryos/larvae, it was important to identify concentrations that directly impair heart contractions, impede biomedical pathways regulating development, and/or cause a general toxicity to all of the cells of the embryo/larvae. Thus, for each cardiac drug, a range of concentrations were tested. An important goal of this testing is to identify a single effective concentration for each cardiac drug that would prevent hearts from contracting and/or perturb embryonic development, but without being too high of a concentration to cause general toxicity/death to the embryos/larvae. Typically, in toxicology/teratogen screening studies, this effective concentration is found at the LD50. Of the surviving group, developmental defects may be observed and in some instances those defects may also be observed in those that do not survive. In the case of this study, death was observed in multiple ways. Death in an early stage of development would be seen as white apoptotic tissue that appears as a ball, which indicates that death occurred early due to the lack of a complex shape. While death in the later stages was assessed by decomposition of the larval tissue, or in some cases full development, but a complete cessation of heartbeat and lack of response to touch.

When the embryos were properly selected, and the controls developed normally, the treated embryos would show a range of phenotypes at the LD50 concentration. This range included anything from alive and healthy looking to being visibly dead. This could likely be explained by the unequal dispensing of the drugs (even though it came from the same working

stock), small variations in the genetics of each of the embryos, or even random chance. If the drug were to cause a developmental defect, it would be the most pronounced at the LD50 concentration, which is a common result in zebrafish toxicology screenings.

As mentioned earlier, LD50 concentrations were assessed at 4 dpf. Initially a broad range of concentrations was used to narrow down the potential LD50 concentrations. Then subsequent to these initial experiments, the range of concentrations tested became smaller until the LD50 was determined. Based off my analysis, the LD50 for verapamil was 10-12.5 µg/ml (Table 1). The LD50 for BDM was determined to be 8-9 mM (808.8-909.9 µg/ml for comparison) (Table 2). Finally, the LD50 for amlodipine was determined to be 9.5-10 µg/ml (Table 3). These concentrations resulted in about half of the embryos dying and half surviving. For each, concentrations above the LD50 typically resulted in greater than 50% of the embryos/larvae dying and at very high concentrations all of the embryos/larvae died.

Verapamil Treatment Resulted in Impaired Heart Contraction

My main drug of interest for the teratogen screening in this study was the calcium channel blocker verapamil. I was interested in verapamil because treatment of zebrafish with a cardiac inhibitor would be more likely to impair heart contraction and possibly lead to impaired heart and blood vessel development. Thus, knowledge of the effective concentrations that lead to impaired embryonic heart contraction in zebrafish may be important in guiding future mammalian studies. Furthermore, an earlier study showed that embryonic exposure to verapamil caused a defect in the development of the jaw (Yamashita et al., 2014). This finding provided evidence that verapamil may act as a teratogen affecting both cardiac tissues and non-cardiac tissues with the latter defect elicited either indirectly via lowered cardiac output or directly via

verapamil's action with these non-cardiac tissues. This further warranted experimentation into the full documentation and quantification of verapamil's induced phenotypes.

There were multiple signs that verapamil exposed larvae lacked heart contraction. For one, based off my observational analysis, a majority of the 4 dpf and 5 dpf larvae had a lack of heart contractions. Moreover, blood flow was absent, as the presence or absence of flow can easily be visualized under the microscope in the vessels of the yolk sac. Also, the presence of edema in the pericardial sac was another indicator that blood flow was absent. Pericardial edema is a common co-morbidity in the lack of blood flow. Pericardial edema is caused by the buildup of fluid between the area outside of the heart and the pericardium (Zakaria et al., 2018). This edema was very prevalent and was observed in almost all of the verapamil exposed larva.

To evaluate if the pericardial edema was statistically relevant, I measured the length of the pericardial sac, starting at the heart, to the edge of the pericardium. For the verapamil groups a total of 26 larvae from 4 independent experiments for 4 dpf, and 20 larvae from 4 independent experiments for 5 dpf were measured for pericardial sac extension and used for statistical analysis. At both 4 and 5 dpf there was a significant increase in the pericardial edema with a mean difference of 144.48 μm at 4 dpf (p-value= 1.62×10^{-10}) and a mean difference of 167.05 μm at 5dpf (p-value= 4.33305×10^{-10}) (Figure 1). These observations confirmed that cardiac function decreased at the LD50 concentration of 10 μg/ml.

Verapamil Treatment Resulted in Impairment of Non-Cardiac Tissues

In addition to cardiac defects, verapamil-treated embryos also showed malformations in tissues other than the heart (referred to as non-cardiac tissues). The most noticeable of these being edema of the yolk sac, a cavity that comprises the intestinal tube, and a very characteristic

dorsal curvature of the tail, which is a phenotype that has not been reported. In addition to these changes, I also observed the jaw defect that was identified in a previous study (Yamashita et al., 2014). Upon visual inspection under the dissection microscope, the jaw appeared to be enlarged and deformed, though it was hard to image with the camera.

Regarding the yolk sac edema, verapamil exposed larvae very commonly exhibited this malformation. This edema was present in almost all of the verapamil exposed embryos at both 4 and 5 dpf. To quantify these results, the distance from the posterior portion of the swim bladder area down to the edge of the yolk sac tissue was measured. For the verapamil groups a total of 31 larvae from 6 independent experiments for 4 dpf, and 25 larvae from 6 independent experiments for 5 dpf were measured for yolk sac edema and used for statistical analysis. At both 4 and 5 dpf there was a significant increase in this edema compared to the control. Four dpf larvae exhibited a mean difference of 128.48 μm (p-value= 1.4528×10^{-11}) and 5 dpf had a mean difference of 286.88 μm (p-value=4.57026×10^{-13}) (Figure 2).

The tail curvature in verapamil exposed larvae was a very characteristic dorsal curvature. The severity of curvature varied between the different larvae, but it did appear to be more severe at 5 dpf compared to 4 dpf. The curvature was measured using the ImageJ angle tool making points along the dorsal side of the embryo. The points were placed in-line with the posterior side of the eye, the posterior side of the gas bladder, and the tip of the tail. For the verapamil groups a total of 28 larvae from 5 independent experiments for 4 dpf, and 31 larvae from 7 independent experiments for 5 dpf were measured for tail curvature and used for statistical analysis At both 4 and 5 dpf, there was a significant increase in the tail curvature compared to the controls. Four dpf with a mean difference of 22.47 degrees (p-value=5.6296×10^{-07}) and 5 dpf with a mean difference of 35.88 degrees (p-value=2.51962×10^{-09}) (Figure 3).

Even though the jaw defect associated with verapamil exposure was apparent by unaided eye under the microscope, it was hard to get an image that would capture this morphological change. As a result, a different approach was implemented to visualize the jaw. At 4-5 dpf, the jaw continues developing but is functional and is made up of developing cartilage (Mork and Crump, 2015). Therefore, if there was a malformation in the jaw, it would be better visualized by doing a stain for the embryonic cartilage. Although I was not able to repeat the study for statistical significance, I was able to see a qualitative difference in the jaw development in verapamil compared to the control (Figure 4). For example, a lateral view of a control larvae at 4 dpf revealed an upper cartilage and a lower cartilage that ran almost parallel to each other. From the ventral view, many branches and arches that the embryonic cartilage forms were apparent (Figure 4). However, 4 dpf verapamil-treated larvae exhibited some differences from the controls. For example, in the lateral view, the lower cartilage appeared to veer downwards in the anterior portion of the head (Figure 4). In addition, there were segments that appeared to not connect well. This indicated that there was a defect in the jaw formation in verapamil exposed larvae.

These data show that verapamil exposed larvae exhibit defects in both cardiac and non-cardiac tissues. It is unclear if these defects are due to the lack of heart function or due to a direct action of verapamil on these non-cardiac tissues.

BDM Impairs Heart Contraction, but Results in Different Non-Cardiac Defects

To assess whether the non-cardiac defects observed in the verapamil treated larvae were due to lack of blood flow (rather than direct action of verapamil), I decided to compare the phenotypes of verapamil to the phenotypes of another kind of cardiac inhibitor called 2, 3-

butanedione monoxime (or BDM). Unlike verapamil, which targets calcium channel blockers and blocks the calcium movement from the sarcoplasmic reticulum into the cytosol, BDM is an ATPase inhibitor that stabilizes the myosin-ADP-Pi intermediate molecule and does not allow for the myosin power stroke to occur (Bond et al., 2013). Similar to verapamil treated larvae, the larvae treated with BDM showed impaired heart contraction and blood flow. To assess BDM's effect on heart development, extent of pericardial edema was analyzed. For the BDM groups a total of 20 larvae from 5 independent experiments for 4 dpf, and 19 larvae from 6 independent experiments for 5 dpf were measured for pericardial sac extension and used for statistical analysis. Treatment with BDM resulted in a significant increase from the controls both 4 dpf and 5 dpf mean difference of 149.91 μm at 4 dpf (p-value= 4.30658×10^{-10}) and a mean difference of 204.73 μm at 5 dpf (p-value=8.63397×10^{-09}) (Figure 1). Congruent with the verapamil data, BDM treatment also resulted in an absence of heart contraction and blood flow, suggesting that even though BDM acts through a different mechanism, it had the same effect on heart function.

If the non-cardiac defects associated with verapamil treatment (yolk sac edema, dorsal tail curvature, jaw defect) were due to lack of blood flow, then I would expect to see the same phenotypes in the larvae treated with BDM. For the BDM groups a total of 18 larvae from 4 independent experiments for 4 dpf, and 21 larvae from 5 independent experiments for 5 dpf were measured for yolk sac edema and used for statistical analysis. Analysis of the yolk sac showed a significant increase compared to controls at both 4 and 5 dpf with a mean difference of 91.75 μm (p-value= 4.22865×10^{-09}) and 218.76 μm (p-value=1.38719×10^{-12}) respectively (Figure 2). Comparing BDM-induced yolk sac edema to verapamil-induced yolk sac edema, there was not a significance difference at 4 dpf, but at 5 dpf there was a significant increase with verapamil when compared to BDM (p-value=0.0174) (data not shown). In regard to the tail curvature, for the

BDM groups a total of 22 larvae from 3 independent experiments for 4 dpf, and 17 larvae from 3 independent experiments for 5 dpf were measured for tail curvature and used for statistical analysis. The BDM treated embryos/larvae did result in a significant increase compared to the controls at both 4 and 5 dpf with a mean difference of 14.29 degrees (p-value=5.67077×10^{-06}) and 11.04 degrees (p-value=0.040669), respectively (Figure 3). However, the tail curvature was different in the case of BDM, with the tail either curving in a wave fashion (down and then up) or the curvature was down. There were only a very few cases in which the tail curved dorsally.

In regard to the jaw defects, 4 dpf BDM larvae did show some difference from the controls. From the lateral view it appeared that there was also a malformation in the lower cartilage. However, in the BDM-treated embryos/larvae, the lower cartilage veers down perpendicular to the upper cartilage, much more extreme than what was observed in verapamil's lower cartilage malformation. Another difference in this lower cartilage defect compared to verapamil is that in the BDM group, the lower cartilage stopped about halfway through the head; whereas, in the verapamil group the lower cartilage continued to the end of the head. Also, the entirety of the BDM exposed embryonic head cartilage appeared to be shorter in length when compared to both verapamil and the control (Figure 4). Though the BDM-treated larvae do share some characteristics with the verapamil-treated larvae, the phenotypes were unique. The BDM treated group did have tail curvature, but it was not the characteristic dorsal curvature exhibited in the verapamil treated group, and the jaw defects observed in the BDM larvae were different than that observed in the verapamil exposed larvae.

Amlodipine Exhibits Impaired Heart Contraction and Very Mild Non-Cardiac Defects

To determine if the verapamil-induced non-cardiac defects were a characteristic of L-type calcium channel blockers, the verapamil-treatment phenotypes was compared to another L-type calcium channel blocker. Initially, embryos were to be treated with nifedipine, a very common cardiac drug and L-type calcium channel blocker. However, despite how popular this drug is used in human and animal studies, use of the drug in this study was reconsidered, as nifedipine needs to be dissolved in an organic solvent such as ethanol or DSMO, and these compounds by themselves have the potential to cause developmental defects. Thus, another L-type calcium channel blocker, amlodipine, was used in this study instead.

Larvae exposed to amlodipine were qualitatively assessed to have low heart function, based on the observation of low heart contractility and lack of blood flow through the visible blood vessels. For the amlodipine groups a total of 20 larvae from 4 independent experiments for 4 dpf, and 10 larvae from 3 independent experiments for 5 dpf were measured for pericardial sac extension and used for statistical analysis. Paradoxically, assessment of pericardial edema at both 4 and 5 dpf revealed that there was not a significant difference from the controls at both 4 and 5 dpf (Figure 1). After many observations it was discovered that embryos exposed to amlodipine had intermittent heart function. Meaning that there were times in which very little or no heart function was observed, and in the same embryos at a different time they appeared to have normal heart function. This intermittent heart function was very sporadic, and for most of the time hearts were not contracting. Thus, the occasional reinstatement of small periods of heart contraction was almost missed.

If the non-cardiac defects associated with verapamil were due to either the indirect effects of low blood flow or the direct effects of L-type calcium channel blockers, then similar

phenotypes should be observed between amlodipine and verapamil-treated larvae. For the amlodipine groups a total of 15 larvae from 3 independent experiments for 4 dpf, and 11 larvae from 3 independent experiments for 5 dpf were measured for yolk sac edema and used for statistical analysis. Amlodipine exposed embryos/larvae were found to have no significant difference in yolk sac edema at 4 dpf as compared to controls, but a significant increase was observed at 5 dpf, with a mean difference of 82.19 μm (p-value=0.0244) (Figure 2). To determine whether the yolk sac edema differed between the verapamil-treated group and the amlodipine-treated group, statistical analysis revealed a significant increase in the verapamil-treated group (p-value=8.69542×10^{-06}) as compared to the amlodipine-treated group (data not shown). This indicates that amlodipine treatment does not have the same effects in regard to yolk sac edema as verapamil treatment.

Tail curvature analysis was also done on amlodipine exposed larvae. For the amlodipine groups a total of 18 larvae from 4 independent experiments for 4 dpf, and 10 larvae from 3 independent experiments for 5 dpf were measured for tail curvature and used for statistical analysis. The results revealed no significant difference between amlodipine-treated larvae with controls at both 4 and 5 dpf (Figure 3). In regard to jaw development, amlodipine embryos at the current LD50 concentration have yet to be stained and imaged, but visual inspection under the microscope reveals no obvious malformations in the jaw tissue. In summary, these results revealed that amlodipine treatment does significantly impair heart contraction (though not a complete absence of heart contraction); however, the treatment does not cause a defect in most non-cardiac tissues such as the jaw or the tail.

Table1. LD50 analysis at 4 dpf for all tested concentrations of verapamil.

Concentration (µg/ml)	Total Alive	Total Dead	Total	%Survivorship
0	129	13	142	91%
5	8	0	8	100%
8	5	3	8	63%
10	114	66	180	63%
12.5	0	13	13	0%

Table 2. LD50 analysis at 4 dpf for all tested concentrations of BDM.

Concentration (mM)	Total Alive	Total Dead	Total	%Survivorship
0	85	11	96	89%
5	22	2	24	92%
6	8	0	8	100%
6.5	23	9	32	72%
7	28	4	32	88%
7.5	23	13	36	64%
8	47	17	64	73%
9	5	7	12	42%
10	6	22	28	21%

Table 3. LD50 analysis at 4 dpf for all tested concentrations of amlodipine

Concentration (µg/ml)	Total Alive	Total Dead	Total	%Survivorship
0	153	12	165	93%
5	30	10	40	75%
6	23	1	24	96%
7	16	0	16	100%
8	17	9	26	65%
9	7	3	10	70%
9.5	57	50	107	53%
10	6	32	38	16%
15	0	16	16	0%
30	0	16	16	0%
35	0	16	16	0%
40	0	16	16	0%
45	0	16	16	0%
50	0	16	16	0%
55	0	16	16	0%

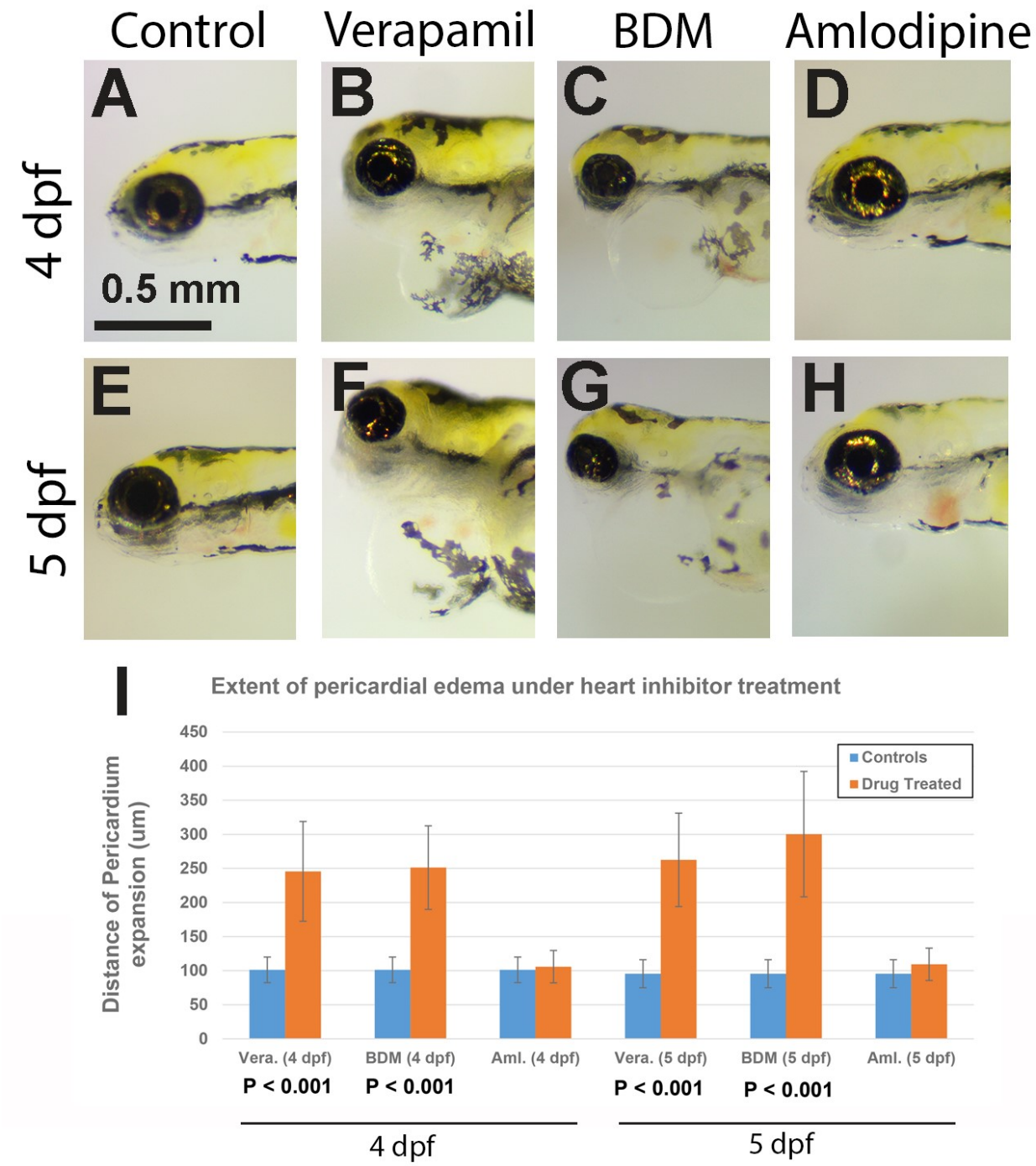

Figure 1. Representative pericardial edema photos at both 4 dpf (panels A-D) and 5 dpf (panels E-H) for the control and all the treatment groups. Panel I shows a graphical representation of the extent of pericardial edema at both 4 and 5dpf. BDM- 2,3-butanedione monoxime.

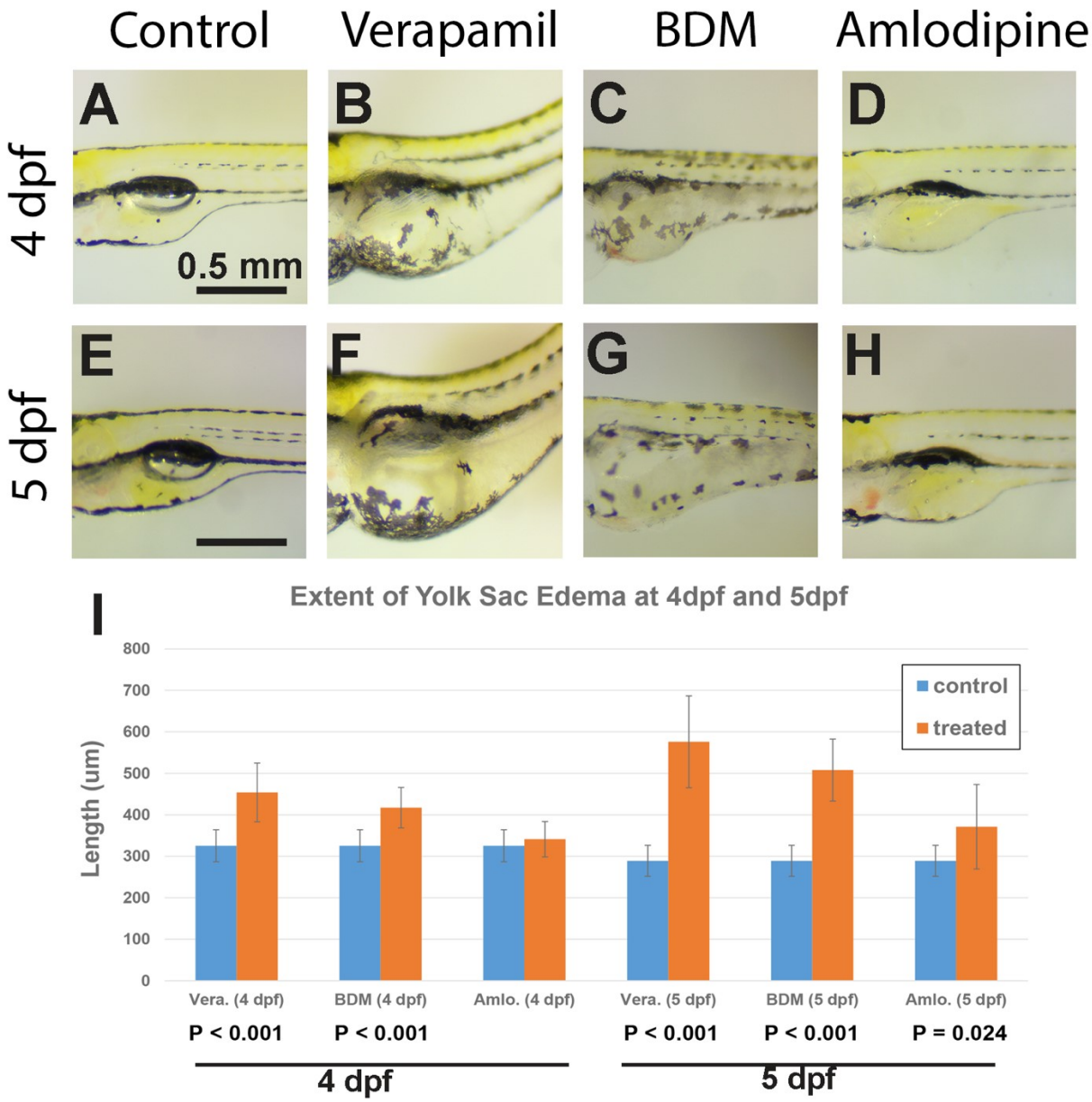

Figure 2. Representative yolk sac edema photos at both 4 dpf (panels A-D) and 5 dpf (panels E-H) for the control and all the treatment groups. Panel I shows a graphical representation of the extent of yolk sac edema at both 4 and 5dpf. BDM- 2,3-butanedione monoxime.

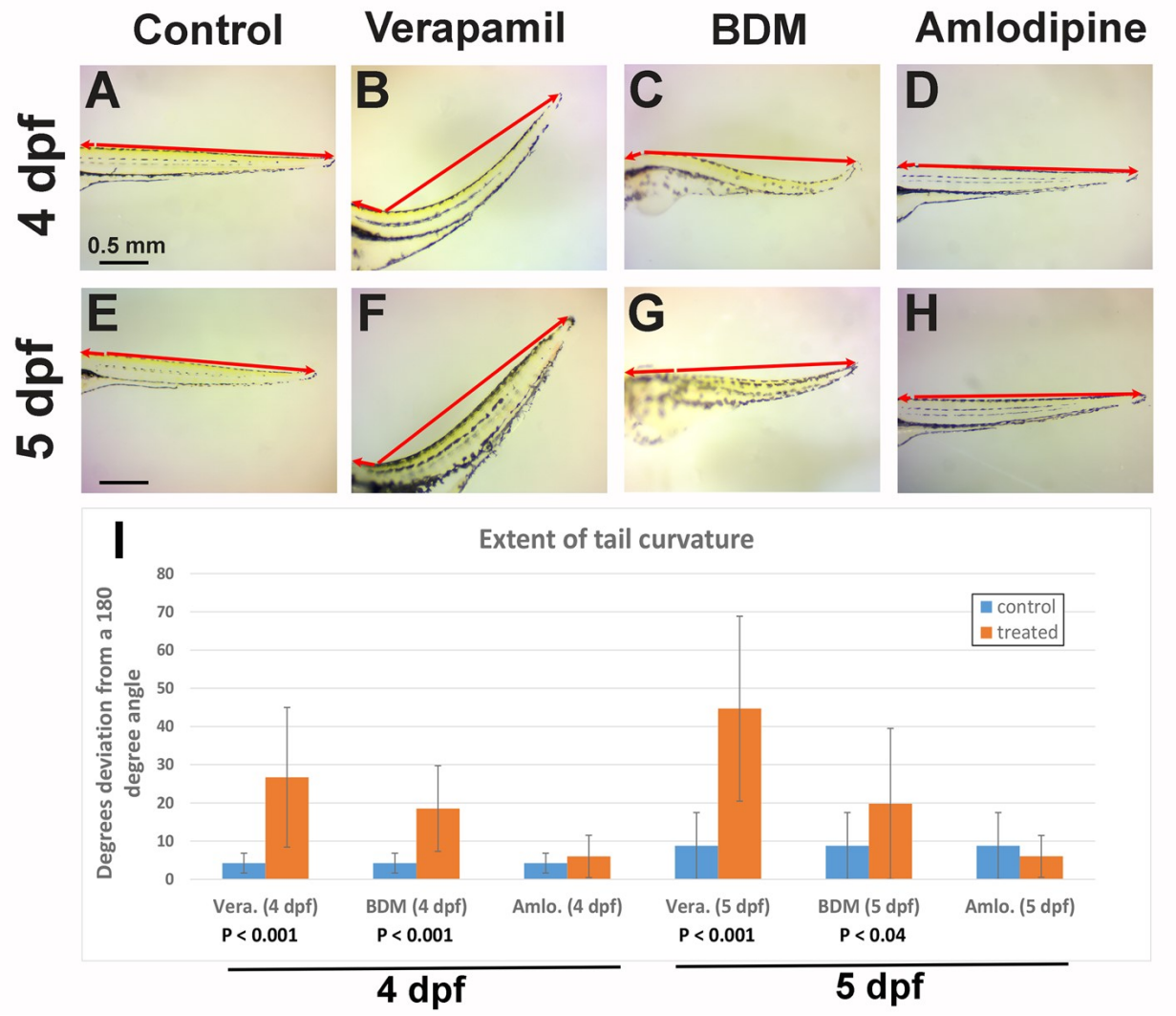

Figure 3. Representative tail curve photos at both 4dpf (panels A-D) and 5dpf (panels E-H) for the control and all the treatment groups. Panel I shows a graphical representation of the extent of tail curvature at both 4 and 5dpf. BDM- 2,3-butanedione monoxime.

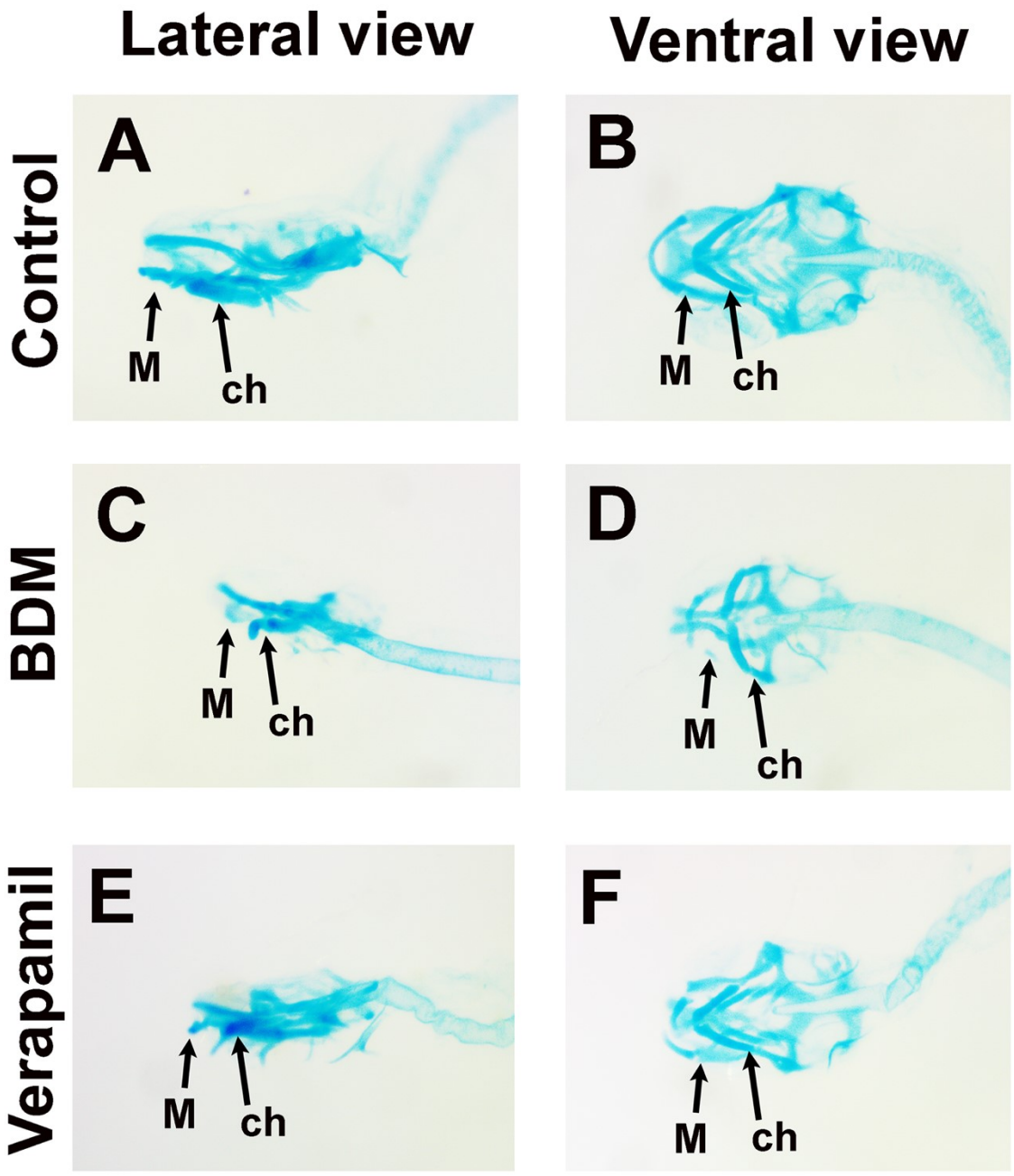

Figure 4. Jaw stain representative images at 4 dpf of control (panels A and B), BDM (2,3-butanedione monoxime; panels C and D), and verapamil larvae (panels E and F).

DISCUSSION

The Importance of Identifying the LD50 and Embryonic Exposure Concentrations of Cardiac Medications

Considering the dangers of pregnancy-induced hypertension, preeclampsia, tachycardias, and fibrillations, it is important that pregnant mothers receive the proper cardiac medication to control blood pressure or other cardiac conditions. However, a concern that needs to be addressed are the potential teratogenic effects of these medications. Between the years 2000 and 2010, there were 172 drugs approved by the FDA. Of these 172 drugs, 168 of them had undetermined teratogenic risk, and 126 had no data about the risk to pregnancy (Stock and Norman, 2019). As a first step to address harmful concentrations that these drugs may pose upon exposure to human embryos/fetuses, use of animal models (such as zebrafish) will allow for the documentation of effective concentrations that may yield a similar teratogenic and cardiotoxic effect in humans. This study has helped to provide that information.

The efficacy of each cardiac medication has to be considered on a case by case basis. This is due to differences in the mechanism of action, solubility, binding strength, and molecular size of each medication. Therefore, the concentration at which each drug is effective was tested. To determine the LD50 of each drug, various concentrations were tested. The LD50 for verapamil, BDM and amlodipine were determined, and were found to exist in a small concentration range. A possible explanation for the range in LD50 for each drug (instead of a specific concentration) was that even though the drugs were kept as solids until the experiments were conducted, there may have been some breakdown of the drugs over time. These LD50

concentrations are now a good starting point by which future studies can conducting in zebrafish and mammalian models.

It is important to compare the LD50 concentration of verapamil to the possible concentration a human embryo would be exposed to during maternal treatment. The transfer of drugs from the mother and across the placenta depends on a lot of factors such as molecular weight, solubility, concentration, rate of excretion from the mother, and rate of detoxification. That being the case, the amount of a drug that reaches an embryo is generally about 30-70% of what is in the mother's plasma (Hill and Kleinberg, 1984). I was not able to find any paper describing the exact concentration of verapamil that makes it to the embryo. However, treatments of IV administered 10 mg verapamil has been used to treat supraventricular tachycardia in pregnant patients (Byerly et al., 1991). That being said, the average pregnant woman has a plasma volume of 3850 ml (Hytten, 1985). Therefore, a theoretical maternal blood concentration of 2.6 μg/ml of verapamil can be deduced. This, in addition to the fact that verapamil has a first-pass metabolism rate of around 80%, suggests that the concentration that an embryo is exposed to may be less, but not far off, than the concentration assessed in this study (Halpern et al., 2019). However, every treatment may be different due to many factors including differing metabolism rate, blood plasma volume, and therapeutic concentrations.

Although the concentration of verapamil that reaches the human embryo is unknown, this study reveals the maximum concentration (LD50) that results in diminished heart function and blood flow, at least in zebrafish. Also, though the effects of these medications are very prominent at the LD50, some effects were observed at lower concentrations. Collectively, these LD50 concentrations may be useful for establishing relevant concentrations in future studies involving mammalian animal models and humans.

The Effect of Cardiac Drug Exposure on Embryonic/Larval Heart Dysfunction and Pericardial Edema

Diminished heart function has been previously shown to cause defects in the heart and associated structures. For example, defects related to heart looping and valve formation have previously been observed in zebrafish with lowered blood flow (Hove et al., 2003). A more obvious defect brought about by low blood flow in this study is the presence of pericardial edema. Pericardial edema is a common co-morbidity of lowered blood flow and is a sign of embryonic heart failure (Chen, 2013). This edema is due to the increase of hydrostatic pressure resulting from heart failure, causing fluid to leak into the pericardial sac (Hanke et al., 2013). It is no surprise that pericardial edema was observed with BDM and verapamil, as animals treated with these drugs were observed to have lowered cardiac function and blood flow. However, an interesting result is the lowered amount of pericardial edema in amlodipine exposed larvae. This less extreme phenotype may be attributed to the fact that amlodipine larvae exhibit intermittent heart function.

Kidney Dysfunction as a Possible Cause of the Verapamil-Induced Yolk Sac Edema

In regard to the yolk sac protrusion observed upon verapamil treatment, I speculate that this was due to fluid buildup in the yolk sac (edema), rather than lack of yolk absorption by the larvae. In a healthy larva, the yolk sac contains an embryonic/larval kidney, and an intestinal tube, and within that intestinal tube resides the yolk nutrients. At 4 dpf the yolk is still being absorbed, but by 5 dpf a majority of the yolk has been absorbed. A comparison of 2-3 dpf wild-type images from other studies to the 4-5 dpf verapamil treated group in this study showed a qualitative increase in size of the yolk sac (data not shown). Therefore, it is unlikely that this

enlargement of the yolk sac was due to lack of absorption of the yolk as it appears to increase more than its normal size. Moreover, the yolk sac edema was more severe in 5 dpf verapamil treated larvae compared to 4 dpf verapamil treated larvae. This further supports fluid buildup as the cause of this enlargement.

Another possible explanation for the enlarged yolk sac is kidney dysfunction. The larval kidney (also known as the pronephros) is important in zebrafish for osmoregulation and water excretion (Outtandy et al., 2019). Consequently, dysfunction in these processes lead to yolk sac edema in zebrafish (Mü Ller-Deile et al., 2019). A proposed model for why yolk sac edema occurs in zebrafish also includes circulatory dysfunction (Hill et al., 2004). However previous studies that have demonstrated lowering heart function via mutations led primarily to pericardial edema, rather than to yolk sac edema (Hill et al., 2004). Thus, the yolk sac edema may be caused partially by lowered heart function in verapamil treated larvae, but perhaps mostly by kidney dysfunction.

Future plans for investigation of possible kidney dysfunction include using a fluorescent dextran clearing assay (Christou-Savina et al., 2015). Comparing the results of verapamil groups and control groups would be of interest to see the difference in kidney function.

Possible Explanation of the Dorsal Tail Curvature

While there are many different types of tail curvature seen in zebrafish embryonic studies, the dorsal tail curvature exhibited by verapamil exposed larvae is of the most interest in this study (Jeanray et al., 2015). Dorsal tail curvature is not the most common phenotype observed in zebrafish studies, but it has been observed in other studies. Perhaps, results from my study may provide insight into how verapamil causes dorsal tail curvature. One example of the

dorsal tail curvature phenotype is upon embryonic exposure to silver dioxide nanoparticles along with decabromodiphenyl ether. This leads to a dorsal tail curvature in 33.9% of the exposed zebrafish (Chao et al., 2017). However, the tail curvature was not as pronounced, and other phenotypes associated with verapamil treatment were not observed. Another example is *in nic*^{b107} mutant fish, which have a mutation causing defective acetylcholine receptors, where the dorsal tail curvature phenotype is observed in about half of the larvae (van der Meulen et al., 2005). In this instance though, the tail phenotype was much more reminiscent to that of verapamil-treated embryos, and again other phenotypes associated with verapamil-treatment were not observed. Mutations in the *polycystin 2* (*pkd2*) and exocyst complex component (*Sec10*) genes similarly give rise to dorsal tail curvature (Fogelgren et al., 2011; Obara et al., 2006). Interestingly, mutations that affect *pkd2* result in phenotypes that most closely resemble verapamil treatment, such as the appearance of pericardial edema, yolk sac edema, and a jaw defect (Obara et al., 2006), suggesting that the action of verapamil may be through Pkd2.

Pkd2 as a Potential Molecular Target of Verapamil to Affect Non-Cardiac Tissues

Whether verapamil-treated phenotypes were caused directly by cessation of heart activity during development or by an additional direct action on non-cardiac tissues could be addressed by comparing phenotypes to other cardiac medications. Though BDM-treatment did block heart function, BDM-treated embryos/larvae exhibited tail curvature phenotypes that were different than those seen upon verapamil treatment. Similarly, BDM treatment caused different jaw malformations. Thus, cessation of blood flow does not explain the exact jaw and tail defects exhibited by verapamil treatment. To support this view, amlodipine-treatment did not cause any tail or jaw defects. Though amlodipine treatment did not result in a complete absence of heart

contraction, qualitative observations revealed that the hearts lacked contractility for significant periods of time. Despite this effect on lowered blood flow, there was no dorsal tail curvature or jaw defects observed, indicating that lowered blood flow does not cause these phenotypes. In support of this notion, mutant zebrafish with impaired heart function also do not show tail curvature or jaw defects.

If lowered blood flow is not responsible for tail or jaw defects, then perhaps L-type calcium channel blockers can directly affect tail and jaw tissue. However, the absence of tail and jaw defects in amlodipine-treated embryos/larvae reveal that L-type calcium channel blockers alone do not cause this defect, suggesting that the actions of verapamil on these non-cardiac tissues must be a unique effect by verapamil.

The similarity between *pkd2* mutant embryos and the verapamil exposed embryos suggests that verapamil may act to block both L-type calcium channels of the heart, and Pkd2 protein channels in non-cardiac tissues. *pkd2* encodes for Polycystin-2, a calcium-dependent non-selective cation channel (Schottenfeld et al., 2007). In support of this hypothesis, *pkd2* is known to be expressed in the notochord, skeletal muscles, pharyngeal cartilage of the jaw, and the kidney which are all tissues affected by verapamil treatment. For example, *pkd2* morpholinos have been shown to induce collagen overexpression in the notochord, and this dysregulation in the extracellular matrix in this region could be a likely explanation for this dorsal tail curvature (Mangos et al., 2010). Alternatively, another explanation for this curvature may be that verapamil decreases muscle activity in the axial muscles, leading to an altered muscle composition. This altered muscle composition can lead to dorsal curvature, as demonstrated with *nic*^{b107}(van der Meulen et al., 2005). Pkd2 is expressed abundantly in the skeletal muscle of zebrafish embryos, so it is possible that verapamil may interact with Pkd2 in the skeletal muscle

to alter muscle activity. Interestingly, verapamil exposed larvae had a lowered response/movement compared to the control larvae (data not shown). So, it is possible that they had lowered muscle activity, which would lead to the altered muscle composition, which in turn leads to the dorsal curvature.

In another example, results from my studies have revealed that verapamil-treated larvae qualitatively showed diminished amount and altered structure of pharyngeal cartilage that forms the jaw. Similarly, other studies have demonstrated that *pkd2* is expressed in the pharyngeal arch cartilage (England et al., 2017) and that mutation of the closely related gene, *pkd1* results in impaired pharyngeal cartilage formation, and thus an abnormal jaw (Mangos et al., 2010). Finally, the observed yolk sac edema in verapamil-treated embryos may be caused by kidney dysfunction. Interestingly, Pkd2 is required for normal kidney function and development, and mutation results in kidney dysfunction (Obara et al., 2006). However, at this point, to determine whether verapamil treatment causes kidney dysfunction has to be validated in future fluorescent dextran clearing studies.

To provide even more evidence to support the hypothesis that verapamil targets Pkd2, future studies will involve testing if verapamil-treated embryos/larvae exhibit defects in left-right asymmetry of the heart, a phenotype also associated with *pkd2* mutants (Schottenfeld et al., 2007). Future experiments could also involve staining of the atria and ventricle of the zebrafish embryos to visualize if the heart loops in the correct way. If verapamil exposed embryos show this reversal of the looping, then this would be further evidence that verapamil interacts with the Polycystin-2 channel.

Though more studies will need to be done to further evaluate whether verapamil acts through Pkd2, whether the LD50 concentrations in this study applies to humans, and whether

human embryos are exposed to LD50 concentrations, finding from my study have provided evidence to warrant that doctors should be cautious when prescribing these medications to pregnant mothers. My findings have demonstrated that treatment with verapamil (and BDM) resulted in more severe phenotypes as compared to amlodipine treatment (Figure 5). BDM is not an FDA approved cardiac drug, so the finding that this showed obvious defects in the embryo is not too concerning. However, the fact that verapamil treatment showed obvious defects is concerning. This evidence suggests that the use of verapamil during pregnancy may be a cause for concern, and perhaps when available, amlodipine may be a better option.

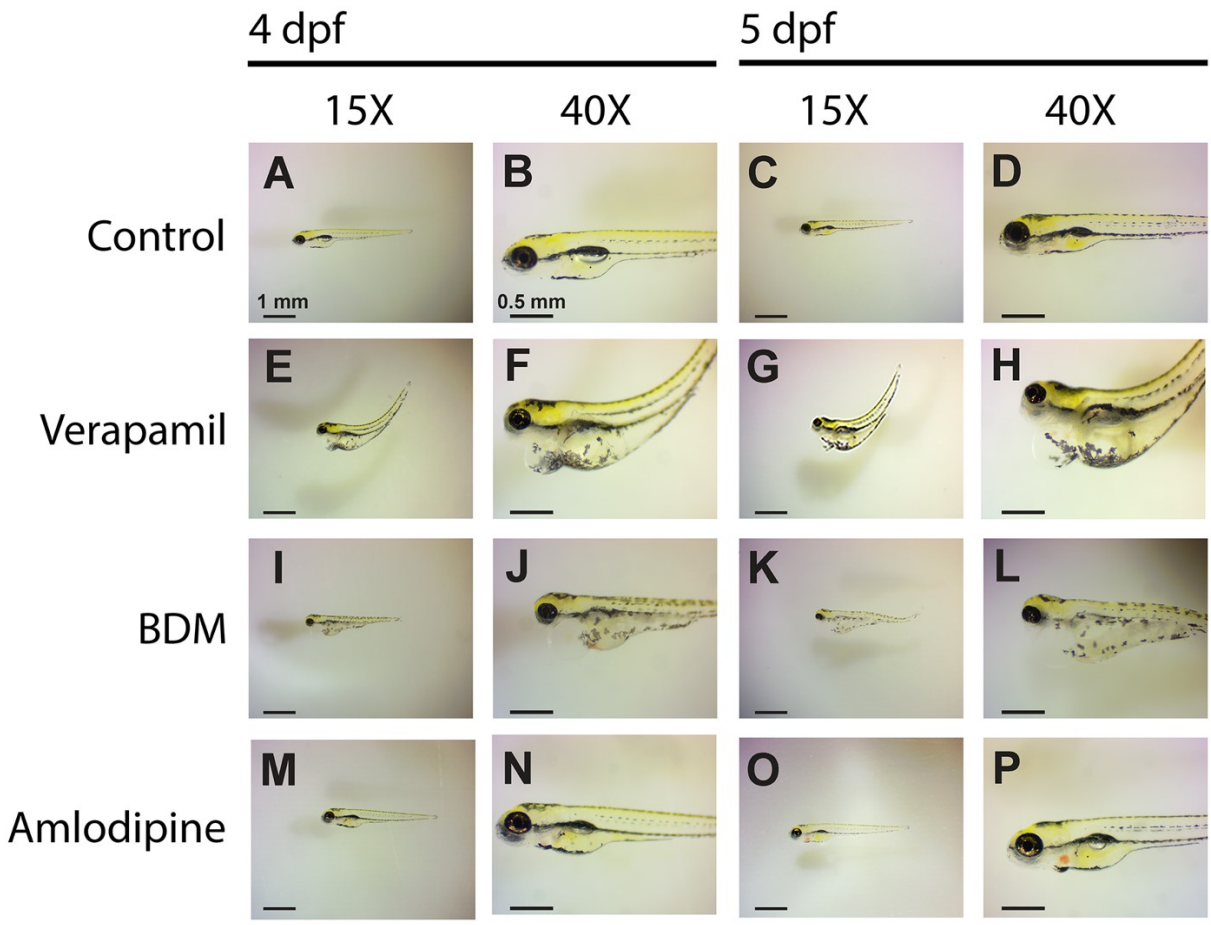

Figure 5. Representative photos at both 15X and 40X magnification at both 4 dpf and 5 dpf for the control and experimental groups. BDM- 2,3-butanedione monoxime.

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