IN UTERO AND POSTNATAL DEFICITS IN RAT CARDIAC FUNCTION FOLLOWING GESTATIONAL EXPOSURE TO DIMETHADIONE, THE N-DEMETHYLATED METABOLITE OF THE ANTICONVULSANT TRIMETHADIONE

by

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ABSTRACT

BACKGROUND: The ventricular septal defect (VSD), a hole between the ventricles of the heart, is the most common birth defect. Despite its commonality, little is known about related in utero functional deficits. Furthermore, although about 80% of clinical VSD resolve within a year, the long-term effects after their resolution are unknown due to lack of clinical follow-up. Chemical treatment was used to induce VSD in the rat and to investigate their functional consequences both in utero and postnatally. METHODS: Pregnant Sprague-Dawley rats were administered six 300mg/kg doses of dimethadione (DMO) by oral gavage every 12 hours beginning at 19h00 on gestational day (GD) 8 (Weston et al., 2011). DMO is the N-demethylated metabolite of the anticonvulsant trimethadione, a potent inducer of VSDs clinically and in laboratory animals. Fetal heart structure and function were examined with high-resolution ultrasound on GD 14, 15, 16, 17, and 21. A separate cohort of rats was dosed using the described paradigm, but offspring were allowed to reach parturition and mature naturally. Postnatal heart structure and function were assessed using telemetry (70 days postnatally), high-resolution ultrasound, and electrocardiography (ECG) (one year postnatally). RESULTS: Relative to controls, DMO-treated fetal rats had structural defects including VSD, an increased incidence of bradycardia (23 vs. 45%) and dysrhythmia (1.2 vs. 11%), and a reduction in cardiac output, stroke volume, and mean heart rate. Adult rats exposed to DMO in utero were more physically active, had elevated blood pressure, and had a higher incidence of dysrhythmia associated with ECG disturbances compared to controls. Both in utero and postnatal functional deficits occurred independent of septum patency. CONCLUSIONS: Gestational exposure to DMO disrupted cardiac function both in utero and postnatally,
even in the absence of gross structural defects, indicating chemical exposures in utero may have permanent pathophysiological consequences on the heart.

**Keywords:** dimethadione, trimethadione, ventricular septal defect, congenital heart defect, dysrhythmia
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<table>
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<th>Description</th>
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<tr>
<td>2D</td>
<td>two dimensional</td>
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<tr>
<td>a</td>
<td>atrium</td>
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<tr>
<td>AM-mode</td>
<td>anatomical mode</td>
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<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ao</td>
<td>aorta</td>
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<tr>
<td>ASD</td>
<td>atrial septal defect</td>
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<tr>
<td>AV</td>
<td>atrioventricular</td>
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<td>avj</td>
<td>atrioventricular junction</td>
</tr>
<tr>
<td>B-mode</td>
<td>brightness mode</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cx40</td>
<td>connexin 40</td>
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<tr>
<td>CHD</td>
<td>congenital heart defect</td>
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<tr>
<td>CTL</td>
<td>control</td>
</tr>
<tr>
<td>DMO</td>
<td>dimethadione</td>
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<tr>
<td>ECG</td>
<td>electrocardiography/ electrocardiogram</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GATA4</td>
<td>zinc finger domain transcription factor 4</td>
</tr>
<tr>
<td>GD</td>
<td>gestational day</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HERG</td>
<td>human ether-a-go-go-related gene</td>
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<tr>
<td>$I_{Kr}/I_{Ks}$</td>
<td>delayed rectifier potassium channels</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<tr>
<td>L</td>
<td>liter</td>
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<tr>
<td>la</td>
<td>left atrium</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>lv</td>
<td>left ventricle</td>
</tr>
<tr>
<td>lvw</td>
<td>left ventricular wall</td>
</tr>
<tr>
<td>M-mode</td>
<td>motion mode</td>
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MAP                   mean arterial pressure
mg                    milligram
MHz                   megahertz
min                   minute
mL                    milliliter
minK                  minimum potassium voltage-gated channel
MLC2v                 myosin light chain 2v
mM                    millimolar
mm                    millimeter
mmHg                  millimeter of mercury
Nkx2.5                homeodomain-containing transcription factor 2.5
NPPA                  natriuretic propeptide A
ot                    outflow tract
pa                    pulmonary artery
PND                   postnatal day
ra                    right atrium
RMV                   real-time microvisualization
rv                    right ventricle
rvw                   right ventricular wall
s                     seconds
sep                   septum
SERCA2a               sarcoendoplasmic reticulum calcium ATPase 2a
sv                    sinus venosa
Tbx5                  T-box transcription factor 5
TMD                   trimethadione
v                     ventricle
VSD                   ventricular septal defect
µg                    microgram
µL                    microliter
µm                    micrometer
CHAPTER 1

INTRODUCTION
1.1 Statement of the Problem

Congenital heart defects (CHD) are the most common anomaly at parturition, accounting for almost one third of all birth defects (Dolk et al., 2011). It is estimated that 1.35 million infants are born with a CHD worldwide every year (van der Linde et al., 2011). CHDs are the leading cause of infant mortality (Rosamond et al., 2008; Bernier et al., 2010), and therefore represent a significant health burden. Our research laboratory is studying the etiology and consequences of CHDs with the ultimate goal of designing intervention strategies to reduce the incidence and deleterious consequences of these defects.

Of the various CHDs, the ventricular septal defect (VSD) is the most common (Rosamond et al., 2008; Bernier et al., 2010), occurring in an estimated 4 out of 1000 live births (Hoffman and Kaplan, 2002). A VSD is a result of failure of the interventricular septum to close completely, causing mixing of oxygen rich blood in the left ventricle with oxygen poor blood in the right ventricle. This state of chronic hypoxia and hemodynamic imbalance can have deleterious effects on the developing fetus (Guissani et al., 2012) leading us to believe that VSD may be associated with in utero deficits in cardiac function. Thus, one aspect of my thesis investigated the functional consequences of structural defects in the fetal rat heart.

VSDs can range in size and severity and although approximately 85 - 90% of these VSDs spontaneously resolve (Roguin et al., 1995; Du et al., 1998), the remaining 10 - 15% of infants with patency of the septum may require surgical intervention (Hoffman and Kaplan, 2002; Asou, 2011). If the VSD is not corrected, there are severe hemodynamic consequences and even following surgery these individuals are still at risk
for severe cardiac pathologies later in life including hemodynamic disturbances and conduction defects (described in Section 1.4.4). In some cases specific gene mutations are believed to cause CHD, which often are associated with the onset of functional pathologies later in life. In these individuals, the development of further cardiac disease may be attributed to the disruption of the gene programs related to the initial structural defect because these same genes control cardiac function postnatally (described in Section 1.4). Thus, all VSD patients may be at risk for heart disease later in life, including patients with spontaneously resolved VSD; however, due to loss of clinical follow-up in this patient group, their risk remains speculative (Meijboom et al., 1994; Pierpont et al., 2007).

To address this important clinical quandary, another goal of this research project was to use an animal model to study the long-term postnatal consequences of VSD on cardiac function in offspring with spontaneously resolved and unresolved VSD. Investigation of this research question is important for several reasons. Firstly, the pathological consequences of VSD may be more insidious in patients with spontaneously resolved VSD because they are unaware of heightened risk, in contrast to patients with unresolved VSD who are monitored for a lifetime. In addition, advances in diagnostic technology and available treatments have increased the survival of patients born with a CHD to the point where there are currently more adults living with a CHD than children (Marelli et al., 2007; Pierpont et al., 2007; Bruneau, 2008; Khairy et al., 2010). As the average lifespan of CHD patients increases, it is imperative that we gain a greater understanding of the postnatal functional consequences of CHD.
1.2 Etiology of Congenital Heart Defects

The etiology of CHD is poorly understood but is considered to be a combination of genetic predisposition and environmental influence (Nora, 1968). Often, CHD exhibits familial inheritance (Holt and Oram, 1960; Schott et al., 1998; Benson et al., 1999), indicating there is a genetic element to the incidence of CHD. Studies of individuals exhibiting different forms of CHD have allowed insight into specific genes that are the causal factors (Pierpont et al., 2007). For example, Holt-Oram Syndrome has been ascribed to both gain- and loss-of-function mutations in the transcription factor, T-box transcription factor 5 (discussed in Section 1.4.1). In spite of the insights into heart development provided by various recognized clinical mutations, the reality is that only approximately 10% of CHD cases can be ascribed to an identifiable genetic abnormality (Hoffman, 1990). The remaining 90% of CHD cases have been linked to a variety of environmental factors including maternal diseases, nutritional status, exposure to pharmaceutical products, recreational drug use, and exposures to industrial chemicals and pesticides (Jenkins et al., 2007). For this reason, we chose to study animals in which VSD was generated by an in utero chemical exposure. Specifically, we treated fetal rats with dimethadione (DMO), the teratogenic metabolite of the anticonvulsant trimethadione (TMD), which is described in full in Section 1.5.

A broad array of highly varied chemicals with divergent pharmacological and toxicological properties induce a constellation of similar heart defects, suggesting the possibility of a common biological target (Weston et al., 2006), the identity of which remains obscure. Elucidating the mechanism by which these diverse chemicals induce CHD is difficult considering the heart is a complex three-dimensional organ that
undergoes a complicated and tightly regulated development process, the highlights of which are described in the following section.

1.3 Overview of Heart Development

Heart development in vertebrates is composed of a series of precisely executed morphogenetic events that begin during gastrulation, making it the first organ to initiate morphogenesis (reviewed in Srivastava and Olsen, 2000; Nemer, 2008). A summary of heart development in the rat is found in Figure 1.1 and described in detail below. The process of heart development in the rat is reflective of that in humans, albeit with differing gestational periods. As a point of reference, gastrulation begins on gestational day (GD) 8.5 – 9.5 in the rat and GD 13 – 19 in humans. Heart development is completed at parturition, which occurs on GD 22 in rats and after 39 weeks in humans (reviewed in DeSesso, 1997). Thus, the vertebrate heart is vulnerable to teratogenic insult for most of gestation.

The initiating event of vertebrate heart development is the migration of myocardial progenitor cells from the anterior lateral plate mesoderm to a region caudal to the head folds on either side of the midline (reviewed in Buckingham et al., 2005; Nemer, 2008). Signalling from the surrounding endoderm induces the migrated cells to commit to the cardiac lineage after which they form the cardiac crescent, known as the first heart field, which occurs at GD 8 in the rat (reviewed in Schultheiss et al., 1995; Srivastava and Olsen, 2000; Buckingham et al., 2005). Fusion of the cardiac crescent along the midline of the body forms the linear heart tube, which begins dysrhythmic contractions between GD 9 and 10 in the rat (reviewed in Srivastava and Olsen, 2000). A second cell
Figure 1.1. Overview of heart development in the rat. Heart development is initiated with the commitment of mesoderm cells to the cardiac lineage which form the cardiac crescent at GD 8. Fusion of the crescent along the midline forms the linear heart tube which begins beating between GD 9 and 10. The linear heart tube undergoes looping between GD 10 and 11, allowing for atrial and ventricular specification. As the heart matures, the cardiac conduction system begins to form and the singular outflow tract septates to form the aorta and pulmonary artery. The interventricular septum begins to form around GD 14 and is complete by GD 16 (arrow head); lv, left ventricle; rv, right ventricle; v, ventricle; la, left atrium; ra, right atrium; a, atrium; ot, outflow tract; ao, aorta; sv, sinus venosa; pa, pulmonary artery. Modified from Bruneau, 2002.
source found both anterior and dorsal to the linear heart tube, known as the second heart field, is recruited to the poles of the linear tube (reviewed in Buckingham et al., 2005). These two separate heart fields give rise to specific myocardial cell lineages: the first heart field goes on to form the entire left ventricle and contributes to the right ventricle and the atria, while the second heart field goes on to form the outflow tract and also contributes to the right ventricle and the atria (reviewed in Buckingham et al., 2005; Bruneau et al., 2008). Thus, both the first and second heart field are vulnerable to conditions that reduce cell numbers or interfere with cell migration within the embryo, and moreover, such disruptions would be expected to have lineage-specific consequences. For example, dosing with DMO at a time corresponding to migration of the second heart field increased the incidence of outflow tract anomalies (Weston et al., 2011).

At approximately GD 11 in the rat, the developing heart enters the looping stage, which through a complex series of events allows atrial and ventricular chamber specification (reviewed in Srivastava and Olsen, 2000). Rightward directed looping is essential for proper alignment of the ventricles with the major vessels of the heart and division of the pulmonary and systemic circulatory systems (reviewed in Srivastava and Olsen, 2000; Buckingham et al., 2005). Each cardiac chamber has individual structural design, contractile properties, and pattern of gene expression (reviewed in Srivastava and Olsen, 2000). It is this stage of heart formation that accounts for the majority of CHDs, including VSD. CHD can result from even a subtle interference with the individual properties of the developing chambers, often due to early disturbances in compartmentalization of the developing regions of the heart (reviewed in Nemer, 2008).
As the ventricles develop, a subset of cardiomyocytes surrounding the developing coronary arteries differentiate into cardiac conduction cells that form the conduction system through the heart (Hyer et al., 1999). Disruption of the differentiation and migration of these conduction cells through the developing heart prevents the complete construction of a functional cardiac conduction system. This defect may not initially be recognized in affected individuals but carries the potential to cause critical issues with the ability of the heart to function. For example, a reduction in the number of cells within the atrioventricular (AV) node has no effect on cardiac performance at parturition or in young animals, but as the animal ages, conduction pathologies arise (Jay et al., 2004). The initially singular outflow tract divides to form the aorta and the pulmonary arteries (reviewed in Srivastava and Olsen, 2000). Failure of this process results in persistent truncus arteriosus, a deleterious CHD due to the consequences of the lack of separation between the pulmonary and systemic circulations. As the heart chambers mature, trabeculation, the process of organization of cardiomyocytes into bundles, occurs along the outer walls of the growing ventricles (Christoffels et al., 2000). Disturbances to the proliferation or the migration of cardiomyocytes at this stage may interfere with the formation of the trabecular structure of the ventricular walls and may result in reduced cardiac output. The second last phase of chamber formation is the growth and migration of the interventricular septum from the apex of the heart toward the AV node. This process is initiated at approximately GD 14 and completed by GD 16 in the rat (reviewed in DeSesso, 1997; Purssell et al., 2012). Thus a patent ventricular septum after GD 16 is referred to as a VSD. Most chemically-induced VSD resolve spontaneously postnatally, leading some to suggest that VSD may not be a frank malformation, but rather a
developmental delay (Fleeman et al., 2004). The last major event in chamber formation is
the closure of the foramen ovale between the left and right atria at parturition. Continued
patency results in an atrial septal defect (ASD).

In summary, the complexity and duration of heart development make it
particularly vulnerable to developmental disturbances and it is therefore not surprising
that CHD is the most common class of birth defect. Moreover, cardiogenesis is
orchestrated by a myriad of genes whose expression may be altered by a variety of
chemical exposures and pathophysiological conditions. Some of the more important
genes related to my work are discussed in the following section.

1.4 The Genetic Link Between Cardiac Structure and Function

As previously described, heart formation is complex and of long duration.
Therefore, the execution of this developmental process requires precise spatiotemporal
signalling from a number of critical genes, the functions of which are conserved across
vertebrate species (reviewed in Srivastava and Olsen, 2000; Nemer, 2008). For example,
the role of the T-box transcription factor, Tbx5 in ventricular septation and patterning is
conserved throughout vertebrate evolution. Mammals possess a four-chambered heart due
to a specific Tbx5 expression pattern (Koshiba-Takeuchi et al., 2009). It is not surprising
that functional mutations of many genes may lead to structural defects of the heart such
as VSD. More recently, it has become apparent that many genes controlling cardiac
structure also regulate cardiac function both in utero and postnatally (reviewed in
Bruneau, 2008; Nemer, 2008). Accordingly, investigation of mutations found both
clinically and in mouse models have demonstrated that CHD rarely occurs in isolation,
but rather is associated with functional anomalies of the heart that present at birth or develop later in life (reviewed in Bruneau, 2008). By extension, it is reasonable to propose that environmental influences altering critical gene expression patterns might also induce CHDs along with associated functional pathologies. Indeed, this is the working hypothesis of our laboratory.

For the purposes of providing the rationale for why VSD may be associated with functional anomalies, I will discuss three major transcription factors important in heart development: the T-box transcription factor, Tbx5; the homeodomain-containing transcription factor, Nkx2.5; and the zinc finger transcription factor, GATA4. Although there are a multitude of genes that are critical to heart development in both rat and human (reviewed in Srivastava and Olsen, 2000), I will be focusing on these three transcription factors for the following three reasons.

Firstly, these transcription factors are considered “master” regulators of cardiogenesis. For example, Nkx2.5 is the earliest molecular marker of commitment to the cardiomyocyte lineage (Harvey, 1996; reviewed in Srivastava and Olsen, 2000). Tbx5 and GATA4 are necessary and sufficient for commitment of pluripotent stem cells to the cardiomyocyte lineage (Ieda et al., 2010).

Secondly, with some degree of overlap, these transcription factors direct the expression of critical gene pathways throughout development important to both the structure and function of the embryo/fetal heart (reviewed in Bruneau, 2008) and postnatal cardiac function (Holt and Oram, 1960; Schott et al., 1998; Garg et al., 2003). Figure 1.2 depicts the downstream targets of each of these transcription factors and their major role in cardiac function.
Figure 1.2. Summary of genetic links between cardiac structure and function. The interactions and functions of important genes discussed in this thesis are depicted. The transcription factors Nkx2.5, Tbx5, and GATA4 interact with one another (horizontal arrows) and are important to the formation of the interventricular septum. These transcription factors also co-regulate the expression of downstream targets (diagonal arrows) that are important for the conduction system, blood pressure regulation, formation of the myocardium, and diastolic function. The functions of these genes are conserved across vertebrate species including both rat and human.
Lastly, unpublished results from the Ozolinš laboratory demonstrate the expression of transcripts for Tbx5, GATA4 (Nkx2.5 has not yet been investigated), and some downstream gene targets of all three transcription factors are significantly altered in embryonic rat hearts 24 hours after the last dose of DMO, the chemical of interest in this thesis. This supports the idea that DMO may adversely affect the cardiac functions controlled by Tbx5, GATA4, Nkx2.5 or their downstream targets.

1.4.1 Tbx5

Holt-Oram Syndrome is a rare autosomal dominant disorder (Holt & Oram, 1960; Gall et al., 1966; Hurst et al., 1991; Basson et al., 1994, 1997) caused by a Tbx5 gene mutation (Li et al., 1997; Basson et al., 1997, 1999). The most common cardiac anomalies found in Holt-Oram patients are septal defects (Holt and Oram, 1960; Gall et al., 1966; Hurst et al., 1991; Basson et al., 1994). Physiologically, Tbx5 expression is found in the left ventricle and the atria, but not in the right ventricle or the outflow tract (Bruneau et al., 1999). In genetically modified mice, both a conditional knockout of Tbx5 in the ventricles and ubiquitous expression of Tbx5 in the entire heart result in failure of the interventricular septum to form (Takeuchi et al., 2003; Koshiba-Takeuchi et al., 2009). Interestingly, VSD has also been identified in “atypical Holt-Oram Syndrome” which is the result of a gain-of-function Tbx5 phenotype (Postma et al., 2008). These previous observations together with our data showing DMO significantly increases Tbx5 expression (Ozolinš, unpublished data) is consistent with the idea that DMO-induced alterations in Tbx5 expression play an important role in VSD etiology. Thus, we believe DMO may also alter functional endpoints controlled by Tbx5 or its downstream targets.
Holt-Oram patients often experience cardiac rhythm disturbances that can be severe including dysrhythmia, atrial fibrillation, AV block (impairment of the electrical conduction between the atria and the ventricles characterized by an increase in the P-R interval on the electrocardiogram), bundle-branch block, and bradycardia (Holt and Oram, 1960; Gall et al., 1966; Basson et al., 1994), potentially leading to cyanosis, congestive heart failure, and can result in sudden cardiac death (Holt and Oram, 1960). The inconsistency in the type and severity of defects noted in Holt-Oram Syndrome has been attributed to the variety of mutations of the Tbx5 gene (Li et al., 1997; Basson et al., 1999). Thus, if Tbx5 expression is altered by environmental influences, its expression patterns and resultant phenotype might also be expected to display some degree of variation.

Mutant mouse models support similar functional roles for Tbx5 in experimental animals. Mice that are haploinsufficient in Tbx5 display conduction defects including sinus pauses and AV block (Bruneau et al., 2001). These functional consequences have been attributed to deficiencies in the expression of genes directly regulated by Tbx5 such as connexin 40 (cx40) (Bruneau et al., 2001), a gap junction protein found in the heart that promotes electrical coupling between myocytes. This gene product propagates electrical impulses through the conduction system of the heart and coordinates myocyte contraction (Kumar and Gilula, 1996; Gros and Jongsma, 1996). It is therefore possible that exposure to DMO may interfere with cx40 expression and cause similar pathologies.

Sarcoendoplasmic reticulum calcium ATPase isoform 2a (SERCA2a) is a calcium pump protein that plays an important role in the removal of calcium from the cytosol of the myocyte to allow diastolic relaxation (MacLennan and Kranias, 2003). Since Tbx5
positively regulates the expression of SERCA2a, Tbx5 mutant mice have impaired diastolic function due to inefficiency of calcium reuptake consistent with a reduction in SERCA2a activity (Zhu et al., 2008). Impaired diastolic function may reduce the ability of the heart to maintain rhythmic contractions and generate force and is an important risk factor for heart failure (Kass et al., 2004). We anticipate that DMO treatment reduces SERCA2a expression leading to poor diastolic relaxation and poor cardiac output.

The expression of myosin light chain 2v (MLC2v) is reduced in the hearts of transgenic mice overexpressing Tbx5 during heart morphogenesis (Liberatore et al., 2000). These transgenic mice had reduced trabeculation (Liberatore et al., 2000), which would be expected to reduce ventricular contractile force. Previous studies indicate that embryonic hearts exposed to DMO also overexpress Tbx5 and have reduced expression of cardiac MLC2v (Ozolinš, unpublished data), further suggesting DMO may impair cardiac output.

Natriuretic propeptide A (NPPA) is also directly regulated by Tbx5 (Bruneau et al., 2001; Ghosh et al., 2001). NPPA codes for atrial natriuretic factor (ANF), a peptide hormone that plays a role in the regulation of fluid and electrolyte balance and blood pressure (Needleman et al., 1985). Tbx5 knockout mice had a 50% reduction in NPPA expression in the heart overall, but had ectopic expression in the right ventricle in utero (Bruneau et al., 2001). Furthermore, ubiquitous expression of Tbx5 in the developing precardiac field and eventually the entire developing ventricle causes ectopic expression of NPPA in the right ventricle in utero (Takeuchi et al., 2003). Similarly, rat embryos exposed to DMO also have ectopic expression of NPPA in the right ventricle (Ozolinš, unpublished data). The basal level of NPPA expression in the adult mutant mice is
comparable to that of the wild-type mice (Bruneau et al., 2001). Nevertheless, it is interesting to speculate whether DMO-induced changes in embryo/fetal expression of NPPA might alter blood pressure in the adult rat or if NPPA expression responds appropriately under stress (ex. salt load) in these animals.

1.4.2 Nkx2.5

As depicted in Figure 1.2, Tbx5 and Nkx2.5 co-regulate many of the same genes. Thus, Nkx2.5 loss-of-function mutations result in cardiac anomalies that are similar to those found in individuals with a Tbx5 mutation (Schott et al., 1998; Benson et al., 1999; Pauli et al., 1999), but additionally may include valve defects (Benson et al., 1999). Like Tbx5, a variety of Nkx2.5 mutations have been discovered that may cause haploinsufficiency or gain-of-function phenotypes (Schott et al., 1998; Benson et al., 1999; Pauli et al., 1999), providing a rational for the variety of types and severity of symptoms observed clinically.

Heterozygous Nkx2.5 knockout mice displayed first degree AV block that presented 7 weeks postpartum as well as abnormalities in conduction through the His bundle and the intraventricular branches (Jay et al., 2004). These functional abnormalities correlated with hypoplasia of the AV node, the His bundle, and the peripheral Purkinje network in the mutant mice (Jay et al., 2004). A ventricular restricted knockout of Nkx2.5 also induced progressive AV block in correlation with hypoplasia of the AV node as well as cardiomyopathy (Pashmforoush et al., 2004). These pathologies have been ascribed to decreases in cx40 expression. Clinically, patients with Nkx2.5 mutations
display similar pathologies (Benson et al., 1999; Pauli et al., 1999; Jay et al., 2004) and are at greater risk for sudden cardiac death (Schott et al., 1998).

Nkx2.5 haploinsufficient mice experienced a downregulation of minimum potassium voltage-gated channel (minK) in the ventricles and the AV node (Jay et al., 2004; Pashmforoush et al., 2004). The minK gene codes for a protein component of the cardiac delayed rectifier potassium channels (I_{Kr} and I_{Ks}) (Sanguinetti et al., 1996; McDonald et al., 1997) that are critical to the process of cardiac repolarization (represented by the Q-T interval on an electrocardiogram) (Li et al., 1996). If DMO were to decrease minK expression, Q-T interval prolongation would be anticipated.

In homozygous Nkx2.5 knockout mice, MLC2v was almost undetectable (Lyons et al., 1995; Tanaka et al., 1999) as was NPPA expression in the ventricles, although it was still found in the atria (Tanaka et al., 1999). Both of these genes are also downregulated in rat embryos after exposure to DMO (Ozolinš, unpublished data) suggesting DMO may also downregulate Nkx2.5 expression and affect a number of cardiac functions, as depicted in Figure 1.2.

1.4.3 GATA4

GATA4 interacts with both Tbx5 and Nkx2.5 to direct heart development (Bruneau et al., 2001; Garg et al., 2003; Takeuchi et al., 2003). Consequently, the clinical phenotype of individuals with a mutation in the GATA4 gene overlaps with that of Tbx5 and Nkx2.5 gene mutations including septal defects as well as valve defects, Tetralogy of Fallot, and cardiomyopathy (Pehlivan et al., 1999; Garg et al., 2003; Nemer et al., 2006), although conduction defects are not always present (Garg et al., 2003).
Existing studies on GATA4 misexpression highlight its critical role in early heart development (Kuo et al., 1997; Molkentin et al., 1997). Studies using mutant mice carrying human GATA4 mutations suggest the variability in the clinical presentation of GATA4 mutations may be linked to the way in which the mutation disturbs the interaction of GATA4 with other transcription factors. For example, in one such mutation, GATA4 interacted with Nkx2.5 normally, but was unable to interact with Tbx5 (Garg et al., 2003). Thus, the significantly increased expression of GATA4 in embryonic hearts after exposure to DMO (Ozolinš, unpublished data) indicates the potential for profound perturbations in the pathways controlled by Tbx5 and Nkx2.5 with high risk for related functional pathologies, as depicted in Figure 1.2.

1.4.4 Functional Consequences of Ventricular Septal Defects

As described in Sections 1.4.1 – 1.4.3, a variety of functional cardiac pathologies are linked to specific gene mutations as a result of shared or common regulatory pathways. Interestingly, although most patients with VSD do not have identifiable mutations in any of the genes described in Section 1.4, they share a number of common functional pathologies (Blake et al., 1982; Kidd et al., 1993; Fukuda et al., 2002; Roos-Hesselink et al., 2004; Walsh and Cecchin, 2007; Liberman et al., 2008; Roos-Hesselink and Karamermer, 2008). This supports our overarching hypothesis which states that a variety of environmental influences alter the expression of the previously discussed pathways.

It is important to note that functional pathologies in patients born with a VSD that does not close may be the result of the shunt between the left and right ventricles. This
will inflict a hemodynamic burden on the right side of the heart that can cause pulmonary arterial hypertension (Duffels et al., 2007). If the pressure in the pulmonary circulation reaches that of the systemic circulation, the flow through the shunt can reverse, allowing deoxygenated blood from the right side of the heart to enter the left side, causing an additional hypoxic burden, a condition known as Eisenmenger’s Syndrome (Engelfriet et al., 2007). Pulmonary arterial hypertension, especially if it progresses to Eisenmenger’s Syndrome, is associated with reduced functional capacity and a significantly higher incidence of patient mortality (Engelfriet et al., 2007).

With the advent of improved therapeutic interventions, it was believed that surgical repair of the VSD corrected the structural defect and related functional consequences; however, epidemiological evidence suggests that all VSD patients are at a greater risk for the development of cardiac pathologies as they age, regardless of whether the VSD persists or was surgically repaired (Kidd et al., 1993; Roos-Hesselink et al., 2004; van der Velde et al., 2005; Liberman et al., 2008; Roos-Hesselink and Karamemer, 2008). Patients that have undergone surgery may still develop pulmonary arterial hypertension and right ventricular hypertrophy, especially when the surgical closure was performed in patients greater than 2 years of age (Pacileo et al., 1998; McLaughlin et al., 2004; Roos-Hesselink et al., 2004). Pulmonary arterial hypertension in patients with a closed septal defect is associated with greater risk of mortality than those with an open defect (Engelfriet et al., 2007), presumably due to a more serious septal defect necessitating the surgical repair procedure. The inability of the hearts to regulate blood pressure in the pulmonary circulation even after surgical correction of the VSD may reflect a developmental disturbance in the expression of a gene that is involved
in blood pressure regulation such as NPPA, a downstream target of all three of the highlighted transcription factors.

The presence of VSD at birth is also associated with the presence of ventricular noncompaction, a rare disease characterized by prominent ventricular trabeculations, deep intertrabecular recesses, and abnormally loose compaction of the endomyocardial walls (Chin et al., 1990; Doğan and Aksoy, 2012). This myocardial phenotype affects the ability of the ventricular walls to generate force and is associated with dysrhythmia, thromboembolic events, and diminished left ventricular systolic function leading to heart failure. The incidence of ventricular noncompaction has been attributed to a disturbance in myocardial morphogenesis (Chin et al., 1990; Doğan and Aksoy, 2012). This phenotype is found in patients with an Nkx2.5 mutation (Pauli et al., 1999) and in fetal rat hearts exposed to DMO during gestation (Weston et al., 2011).

Many VSD patients develop cardiac conduction defects, regardless of whether the VSD resolves (Blake et al., 1982; Kidd et al., 1993; Fukuda et al., 2002; Roos-Hesselink et al., 2004; Walsh and Cecchin, 2007; Liberman et al., 2008; Roos-Hesselink and Karamermer, 2008), including sinus node or AV node block, which cause both supraventricular and ventricular dysrhythmias. As patients age, the prevalence of dysrhythmias becomes greater (van der Velde et al., 2005) and some of these patients may require pacemaker implantation later in life. These patients are at greater risk for sudden cardiac death (Blake et al., 1982; Silka et al., 1998; Roos-Hesselink et al., 2004). These consequences are reminiscent of the conduction pathologies associated with Tbx5, Nkx2.5, and GATA4 mutations, providing further evidence that environmental factors may mediate their deleterious effects by disrupting these pathways.
1.5 The Model

Recall that only approximately 10% of the clinical incidence of VSD can be ascribed to identifiable genetic mutations (Hoffman, 1990). The remaining 90% of VSDs are attributed to environmental influence on the developing heart. The rising incidence of VSD has been proposed to be partially due to the increase in human exposures to drug and environmental contaminants (van der Linde et al., 2011). In light of the significance of environmental contaminants, the use of a chemically induced model is a clinically relevant approach for the study of VSD.

With this in mind, the overarching strategy of our laboratory is to understand how a number of prototypic chemicals, representative of several chemical classes to which humans are frequently exposed, increase the risk of clinical VSD. These include unintentional exposures to industrial solvents and pesticides as well as deliberate exposures to recreational drugs and pharmaceutical therapies for depression and seizure disorders. Chemicals known to increase the risk of VSD in humans and animal models that are of interest to this laboratory include the industrial solvent ethylene glycol (Hanley et al., 1984), the pesticide nitrofen (Kim et al., 1999), ethanol (Bruyere and Stith, 1993; Burd et al., 2007), analgesics (Gupta et al., 2003), and anticonvulsants such as valproic acid (Wu et al., 2010) and trimethadione.

As previously mentioned in Section 1.2, the chemical of interest in my thesis project is dimethadione (DMO), the teratogenic N-demethylated metabolite of the anticonvulsant trimethadione (TMD; Figure 1.3) (Butler et al., 1952). TMD was removed from the market because of its potent teratogenicity; specifically, TMD induced a high
Figure 1.3. Biotransformation of trimethadione (TMD) to dimethadione (DMO) by various maternal CYP P450 enzymes. The font size reflects the relative contribution of these CYP isoforms to the N-demethylation reaction.
incidence of VSD in infants (Zackai et al., 1975; Feldman et al., 1977; Rischbieth, 1979) and in laboratory animals (Solomon et al., 1997; Fleeman et al., 2004).

TMD is N-demethylated by maternal cytochrome P450 enzymes (CYP2E1, CYP3A4, and CYP2C9; listed by relative contribution) to produce DMO (Butler et al., 1952). We decided to dose directly with DMO for several reasons. Firstly, it is the primary source of the teratogenic effects of the drug (Buttar et al., 1978; Wells et al., 1989; Azarbayjani and Danielsson, 1998). Specifically, DMO induces a greater incidence of VSD than TMD when directly compared (Buttar et al., 1978). Secondly, direct administration of DMO removes the potential for variability in the biotransformation of the parent compound. Thirdly, the drug exposures obtained in the rats with our dosing regimen (Weston et al., 2011) reflect those administered to patients when TMD was used clinically. For example, patients on TMD therapy had clinical concentrations of DMO ranging from 200 – 1000µg/mL (Booker and Darcey, 1971). About four hours after the final dose of DMO on GD 11 in the rat model, maternal DMO concentrations are between 1250 and 1900µg/mL, with a mean value of 1600µg/mL, just above the upper limit of pharmacological efficacy (Ozolinš, unpublished data). Lastly, \textit{in utero} TMD treatment induces only membranous VSDs in rats (Solomon et al., 1997; Fleeman et al., 2004), whereas DMO treatment results in the induction of both membranous and muscular VSDs in rats, which reflects the broader localization of VSD noted clinically (Weston et al., 2011). In view of the greater frequency and severity of defects induced, which more accurately reflect the human disease profile, we decided to directly administer the proximate metabolite, DMO.
Several mechanisms have been proposed to explain TMD teratogenicity. The Danielsson group proposed that the teratogenicity may be due to the ability of TMD to inhibit the human ether-a-go-go-related gene (HERG) potassium channel that is responsible for the repolarizing $I_{Kr}$ current (Charpentier et al., 2010). DMO-mediated HERG channel inhibition induced embryonic bradycardia and incidences of dysrhythmia, resulting in periodic hypoxia and reperfusion injury (Azarbayjani and Danielsson, 1998, 2002). The Wells group proposed that DMO is bioactivated by prostaglandin synthetase to form a reactive free radical intermediate (Wells et al., 1989). Our own laboratory has found that DMO disrupts the expression of a number of genes involved in cardiogenesis (Section 1.4). Additionally, we have demonstrated that alterations in gene expression may be the result of the ability of DMO to inhibit histone deacetylase (HDAC) at pharmacologically relevant concentrations (Ozolinš, unpublished data). Most likely, all these proposed mechanisms act in concert to induce CHD.

In light of these observations, we have developed a DMO dosing regimen to maximize the incidence and severity of VSDs, while simultaneously minimizing maternal and fetal toxicity (Weston et al., 2011). The dosing paradigm consists of six 300mg/kg doses of DMO administered by oral gavage (60mg/mL) to the pregnant rats every 12 hours beginning at 19h00 on GD 8. This dosing window reflects a critical period of heart morphogenesis during which the cardiac crescent develops into the linear heart tube and subsequently undergoes looping to become the four-chambered heart (Sissman, 1970). It is an excellent model for five major reasons: (1) our findings are likely to be clinically translatable as TMD is a potent inducer of VSD in humans (Feldman et al., 1977; Rischbieth, 1979); (2) it is an efficient model for the study of VSD as it induces a high
incidence of VSD, approximately 75% (Weston *et al.*, 2011); (3) it induces both membranous and muscular VSDs, as well as additional cardiac structural defects including outflow tract anomalies, reflecting the clinical situation (Weston *et al.*, 2011); (4) the rate of postnatal spontaneous closure of DMO-induced VSD reflects the clinical rate of approximately 80% (Fleeman *et al.*, 2004); (5) it induces gene expression changes that are consistent with rodent and clinical mutations that result in VSD (described in Section 1.4) (Ozolinš, unpublished data).

### 1.6 Research Hypotheses and Objectives

Clinical cases and animal models have clearly demonstrated that mutations of critical cardiac transcription factors give rise to structural and functional deficits of the heart, both in utero and postnatally (described in Section 1.4). Interestingly, only 10% of clinical VSD is attributable to specific gene defects with the remainder being due to environmental influences (described in Section 1.2). That VSD of unknown etiology exhibits many of the functional anomalies seen in mutational models suggests that these environmental perturbations disrupt the same cardiogenic programs required for heart development. This may explain why patients born with persistent VSD, even if surgically repaired, are at increased risk for cardiac pathologies later in life. For the same reason, we posited that patients with a spontaneously resolved VSD are also at risk for cardiac pathologies later in life. Using the previously described DMO-induced VSD model to generate rats with permanent and resolving VSD, I tested the following hypotheses, as outlined in Figure 1.4.
Figure 1.4. Summary of the hypothesis. The hypothesis to be tested is that DMO treatment during a critical window of heart development known to induce a high incidence of VSD will cause functional deficits in utero including a reduction in cardiac output and mean heart rate and an increased incidence of dysrhythmia. In addition, we hypothesize there will be persistent functional deficits in adult animals irrespective of whether the VSD resolves spontaneously or persists. We predict a reduction in cardiac output, an elevation in blood pressure, and an increased incidence of dysrhythmia.
**Hypothesis One:**

Maternal treatment of rats with clinically relevant exposures to DMO will cause persistent deficits in fetal rat cardiac function.

**Objective:** Assess the effect of *in utero* DMO exposure on embryo/fetal rat cardiac function with respect to contractile strength, heart rate, and rhythm.

**Hypothesis Two:**

Adult rats exposed to DMO during gestation will exhibit persistent deficits in postnatal cardiac function.

**Objective:** Assess the effect of *in utero* DMO exposure on adult rat cardiac function with respect to contractile strength, blood pressure, and heart rate and rhythm.
CHAPTER 2

NON-INVASIVE HIGH-RESOLUTION ULTRASOUND REVEALS STRUCTURAL AND FUNCTIONAL DEFICITS IN DIMETHADIONE-EXPOSED FETAL RAT HEARTS IN UTERO.


*Figure 2.2 from this publication was selected for the cover of Birth Defects Research (Part B): Developmental and Reproductive Toxicology 95 (1)
ABSTRACT

BACKGROUND: We previously showed dimethadione (DMO), the N-demethylated metabolite of the anticonvulsant trimethadione, induces ventricular septal defects (VSD) and other heart anomalies in rat (Weston et al., 2011). Because of the relationship between cardiac structure and function, we hypothesized that DMO-induced structural defects of the heart are associated with in utero functional deficits. To test the hypothesis, the goals were (1) define the parameters for ultrasound in the rat conceptus, and; (2) use ultrasound to identify structural and functional deficits following DMO treatment.

METHODS: Different ultrasound modes (B-mode, M-mode, and Pulse-wave Doppler) using four high-resolution ultrasound transducer heads of varying frequency (25 – 40MHz) were tested on gestational days (GD) 14, 15, 16, 17 and 21. Having identified the optimal conditions, pregnant Sprague-Dawley rats were administered six 300 mg/kg doses of DMO every 12 hours beginning at 19h00 on GD 8 to generate conceptuses with a high incidence of VSD. RESULTS: The three ultrasound modalities were used to identify VSD and several novel and rare structural heart anomalies (cardiac effusions and bifurcated septum) in live rat fetuses. DMO-treated hearts had an array of functional deficits including a decrease in mean heart rate, ejection fraction, and cardiac output and increased incidence of bradycardia and dysrhythmia. CONCLUSIONS: The ultrasound biomicroscope is an effective tool for the real-time characterization of the structure and function of embryo/fetal rat hearts. DMO causes significant deficits to in utero heart function for up to ten days (GD 21) following its final administration, suggesting long-term or possible permanent changes to cardiac function.
INTRODUCTION

Ventricular septal defects (VSDs) are the most common cause of congenital heart disease in humans (Hoffman & Kaplan, 2002) and are also a common defect in experimental animals after exposure to various chemicals (Bruyere & Stith, 1993; Sonoda et al., 1993; Gupta et al., 2003; Rufer et al., 2009). To further explore the etiology of chemically-induced VSD, we developed a rat VSD model using dimethadione (DMO), the teratogenic N-demethylated metabolite of the anti-seizure medication, trimethadione (Weston et al., 2011). Dosing every 12 hours from 19h00 on gestational day (GD) 8 to 07h00 on GD 11 with 300 mg/kg DMO induces a mix of axioskeletal and cardiac defects including membranous VSD (68%), muscular VSD (9%) and outflow tract anomalies (Weston et al., 2011). The broad mix of cardiac defects induced by DMO and the fact that its parent compound, trimethadione, was removed from clinical use largely due to the risk of VSD and other heart anomalies after in utero exposure (Zackai et al., 1975; Feldman et al., 1977; Rischbieth, 1979) suggest DMO is a useful tool to study the effects of chemically induced heart anomalies.

VSD is a structural defect, allowing the mixing of oxygenated and deoxygenated blood, and consequently it may also have functional consequences to both the developing heart and the peripheral regions of the embryo. At the histological level, in utero exposure to DMO can result in a myocardium with a “sponge-like,” rather than the more densely compacted appearance (Weston et al., 2011), which would be expected to result in poor cardiac contractility. In addition, mutant mouse models as well as clinical investigations suggest many of the genes that control the development of structures such as the septum (including the T-box transcription factor 5 [Tbx-5] and the homeodomain-
containing transcription factor Nkx2.5) also play critical roles in controlling cardiac functionality including contractility and rhythmicity (Pashmforoush et al., 2004; Zhu et al., 2008). This suggests chemically induced structural defects such as VSD may also co-express with functional deficits such as poor cardiac performance and dysrhythmia. Taken together, these factors prompted our interest in determining whether in utero exposure to DMO, in addition to causing significant structural anomalies that we have previously reported (Weston et al., 2011), also induces functional deficits in the fetal heart.

Ultrasound has been used clinically for decades to characterize cardiac structure and function in both adults and fetuses. These clinical systems operate at frequencies ranging between 2 and 17 MHz and while they have deep tissue penetration, they also have relatively low resolution. Nevertheless, lower frequency ultrasound has been used to garner useful information about embryo/fetal hearts of small laboratory animals. High-resolution ultrasound systems (20 – 55 MHz frequency) have been designed in the last 10 years with reported resolutions of about 30 µm for real-time scanning (Foster et al., 2002; Zhou et al., 2002). The higher frequency allows for more comprehensive examination of fetal heart structure in small animals; however, with the increased frequency and resolution there is a significant decrease in tissue penetration (depth of focus) (Spurney et al., 2006). Thus, one of the challenges of ultrasound is to optimize the trade-off between the depth of focus and the image resolution. At the time this study was initiated, the use of high-resolution ultrasound in the pregnant rat had not been reported; consequently, one of our goals was to determine empirically which scanheads were best suited for the assessment of fetal rat cardiac structures at several time points during
gestation. Furthermore, the externalization of the uterus has been reported to simplify ultrasound examination of mouse embryonic heart structure (Zhou et al., 2002; Spurney et al., 2006). Since rat conceptuses are found in a significantly broader range of tissue depths within the maternal abdomen in comparison to mice, exteriorization would be a decided advantage. Therefore we determined whether or not externalization would be useful for ultrasound assessment of cardiac structure in the rat fetus.

Traditionally, changes in the size or morphology of the embryo or specific organs such as the heart are determined by harvesting the conceptus. Unfortunately, this results in the termination of the fetus and precludes the conduct of longitudinal studies. The non-invasive nature of ultrasound theoretically allows imaging at multiple time points. Using this approach, litters of mice were recently followed longitudinally to generate a database containing various growth parameters (e.g. crown-rump length, cross sectional area, and heart ventricular dimensions) for the developing mouse embryo during gestation (Yu et al., 2008). A further goal of this study was to determine if ultrasound could be similarly used in rats to assess fetal cardiac structure at multiple points throughout gestation and specifically, whether the development of the interventricular septum could be monitored longitudinally in utero.

Ultrasound may also be used as a non-invasive technique to investigate in utero cardiac function in small experimental animals. Yu et al., (2008) used the clinical ultrasound system to assess functional endpoints such as heart rate and contractility parameters (inflow and outflow velocities, contraction and relaxation time, and myocardial performance index) for the developing mouse embryo during gestation. In spite of the fact that the rat is a commonly used species in teratology studies, there is a
relative dearth of information concerning normal in utero rat cardiac function. Thus, another goal of this study was to use high-resolution ultrasound to assess normal fetal rat cardiac functional parameters at selected time points in gestation, and to determine whether a treatment regimen known to induce a high number of cardiac malformations would be associated with decreased in utero cardiac function. Overall, this study will determine the usefulness of high-resolution ultrasound to assess the structure and function of hearts of both physiologically normal and teratogen-exposed rat fetuses.

MATERIALS AND METHODS

Animals

Time-mated Sprague-Dawley rats [Crl:CD(SD)] were obtained from Charles River Inc. (either Kingston, NY or St-Constant, QC). The morning after copulation was designated as GD 0. Upon arrival, rats were housed individually in polycarbonate or polypropylene shoebox cages with heat-treated hard wood chip contact bedding (Sanichips®, P.J. Murphy Forest Products, Montville, NJ or Beta chips®, Northeastern Products Corp. Warrensburg, NY). Environmental room conditions had design specifications as follows: minimum of 12 air changes per hour with air filtered through at least 90% - 95% efficiency filters and then through HEPA filters, relative humidity of 20 – 50% depending on the season, temperature of 70 ± 5°F, and a 12 hour light/dark cycle (lights on/off 07h00/19h00). Certified Rodent Diet 5001/5002 (PMI Nutrition International, LLC, Richmond IN) and drinking water from a municipal source and further purified by reverse osmosis, were provided ad libitum. All procedures underwent veterinary review and were approved either by Pfizer’s Institutional Animal Care and Use
Committee or the Queen’s University Animal Care Committee. 30 dams were examined at Pfizer (Groton, CT) and 28 dams at Queen’s University (Kingston, ON).

**Chemicals**

Dimethadione was purchased from Sigma Aldrich Inc. (St. Louis, MO). Isoflurane was purchased from Pharmaceutical Partners of Canada Inc. (Richmond Hill, ON).

**Treatment**

The day prior to the initiation of dosing, dams were separated into two groups: (1) control and (2) DMO-treated. Separation was based on body weights to ensure the mean body weights of each group were as closely matched as possible. Beginning at 19h00 on GD 8, DMO-treated dams were dosed every 12 hours a total of six times by oral gavage (5mL/kg dose volume) with 300mg/kg of DMO (60 mg/mL drug solution). Control dams received an equivalent volume of distilled water. This treatment regimen was used because we have previously shown it produces a high (74%) incidence of VSD with little accompanying maternal toxicity (Weston *et al.*, 2011).

**Histology**

Rat hearts were removed and placed into 10% neutral buffered formalin for fixation for at least 48 hours. Hearts were then placed into cassettes for processing. The tissues were processed overnight through alcohol and xylene into paraffin using a Shandon ® Pathcentre Tissue Processor. Tissues were embedded in Paraplast Plus™—
hearts were oriented to facilitate cutting from the ventral to dorsal side. Sections were cut 5 µm thick on a microtome and the slides were stained with haematoxylin and eosin (H & E) on a Shandon® automatic slide stainer.

**Ultrasound procedure**

The high-frequency ultrasound imaging systems used in this study were the Vevo660 (Pfizer) and the Vevo770 (Queen’s University) (both from VisualSonics Inc., Toronto, ON). The dams were anaesthetised using a VisualSonics™ isoflurane vaporiser (VisualSonics Inc., Toronto, ON). Anaesthesia was induced by administering 4 to 5% inhaled isoflurane gas in 100% oxygen at 1L/min flow rate to the dams in a small chamber. The plane of anaesthesia was maintained with 2 to 3% inhaled isoflurane gas in 100% oxygen at 1L/min flow rate through a nose cone. The dam was positioned supine on a heating pad and the paws were taped down. The temperature of the dam was maintained at 37°C and the heart rate of the dam was monitored. All procedures were conducted at a maternal heart rate of more than 320 beats/min. Abdominal hair was removed using depilatory cream (Nair®, Church and Dwight Co. Inc, Princeton NJ). Pre-warmed ultrasonic gel was layered over the dam’s abdomen for in situ imaging. In instances where ultrasound was conducted on externalized fetuses (a terminal procedure), a laparotomy was conducted and the gel applied to the outer surface of the uterus. Dams were kept under anaesthesia for no longer than 45 minutes and assessed on no more than two different gestational days to minimize cumulative maternal and fetal stress.

The high-resolution ultrasound biomicroscope uses ultrasound waves variably in order to assess different properties of the fetal heart. Brightness mode (B-mode) produces a 2-dimensional (2D) cross-sectional structural image by lining up numerous scan lines of
the scanning plane and because images are taken every few milliseconds it shows the
real-time motion of the tissue (Pichamuthu, 2009). In this study, B-mode was used to
view the chambers and septum of the fetal heart.

Motion mode (M-mode) is used to quantitatively assess the motion of the heart
(either away from or toward the scanning plane) at a single locus over time. It has greater
temporal resolution than B-mode so it is useful for the detection of rapid movement
(Pichamuthu, 2009). M-mode can only be taken in a strictly vertical plane. If the
ventricles are not aligned appropriately, anatomical M-mode (AM-mode) can be used to
analyze motion along an angled plane; however, because it yields a “rendered” image
AM-mode has less resolution than true M-mode (Pichamuthu, 2009). Specifically, M-
mode and AM-mode were used to assess the contractile force generated by the fetal
hearts based on the distances between the ventricular walls in diastole and systole.
Furthermore, M-mode was used to confirm the presence or absence of a ventricular
septum.

By releasing pulsed waves and measuring the time for the wave reflection to
return to the transducer, Pulse-wave Doppler mode assesses the direction and velocity of
blood flow (Pichamuthu, 2009). Pulse-wave Doppler mode was employed by placing the
probe over the aortic arch to assess heart rate and ejection velocity of the fetal hearts.
Additionally, it was used over the septal region to determine whether there was
bidirectional flow, indicative of a breach between the left and right ventricles.

The real-time micro visualization (RMV) scanheads used in this study included
The centre frequency and optimal focus depth of these scanheads are depicted in Figure.
1. All structural images (except Figure 1) were obtained with the RMV-712 and all functional assessments were obtained using the RMV-707B.

**Data collection**

Ultrasound data was collected on GD 14 (the start of septation), 15 (the middle of septation), 16 (when septation should be completed) (Sissman, 1970; DeSesso, 1997; Laffin et al., 2004), 17, and 21 (day prior to parturition). The conceptuses were located using B-mode and a B-mode scan was made of the embryonic heart. M-mode images were taken across the ventricles to determine the presence or absence of a ventricular septum at a location of a suspected VSD and for assessment of cardiac contractility. Pulse-wave Doppler flow was recorded over the aortic arch to assess heart rate and ejection velocity or over the presumptive septum to determine whether there was flow between the left and right ventricle. Four embryos were imaged per dam.

Some of the dams were sacrificed following ultrasound and the fetal hearts removed, fixed, sectioned and processed with H & E staining to confirm the presence of structures of interest and investigate histological structure.

**Data Analysis**

The ultrasound data was analyzed using the VisualSonics™ software. B-mode was used to assess the patency of the ventricular septum on GD 14, 15, 16, 17 and 21. To confirm patency of the septum, the B-mode images were compared with the M-mode/AM-mode images collected across the ventricular walls of each individual heart.
Further confirmation was conducted using Pulse-wave Doppler by looking for evidence of bidirectional blood flow in the region of the suspected ventricular septation defect.

An M-mode/AM-mode trace of the contracting ventricular walls allows the software to measure the left ventricular volume and diameter of each fetal heart. These measurements were used to calculate stroke volume (µL), ejection fraction (%), fractional shortening (%), and cardiac output (mL/min) of each fetal heart. Stroke volume is the volume of blood pumped out of the ventricle with each beat (difference between the volume in systole and diastole). Ejection fraction is the fraction of the end-diastolic volume in the ventricles that is ejected with each beat (percent difference between the left ventricular volume in systole and diastole as a fraction of the volume in diastole). Fractional shortening is the fraction of the diastolic dimensions of the ventricles that is reduced in systole (percent difference between the left ventricular diameter in systole and diastole as a fraction of the diameter in diastole). Cardiac output is the volume of blood pumped out of the heart per minute (stroke volume multiplied by the heart rate determined using Pulse-wave Doppler). The values for each of these parameters were compared across gestational day and between the DMO-treated and control groups using two-way analysis of variance (ANOVA) with Tukey post hoc tests.

Heart rate (beats/min) and ejection velocity (mm/s) were calculated from Pulse-wave Doppler images. Using the software embedded in the ultrasound system, heart rate was determined peak-to-peak and averaged over a period of 10 seconds. Ejection velocity was determined by the height of each peak and averaged over 20 pulses. The values of each of these parameters were compared between gestational days and treatment groups using two-way ANOVA with Tukey post hoc tests. A dysrhythmia was defined as a heart
rhythm with a standard deviation greater than 5% of the average heart rate of the fetus. Bradycardia was defined as a heart rate that was lower than 10% below the average heart rate for the control group on the specific gestational day. The incidence of bradycardia and dysrhythmia were compared between the DMO-treated and control groups using the Fisher’s Exact Test.

Before beginning the analysis, we investigated if any conditions were interfering with the data collection. Data from dams that were administered greater than 3% maintenance isoflurane through the nose cone were excluded from analysis.

RESULTS

Optimizing conditions

The first step of this study was to identify the optimal conditions for use of the high-resolution ultrasound biomicroscope in rat fetuses. In particular, the effect of two parameters, namely (1) the transducer type, and (2) the effect of exteriorization of the uterus, were examined.

For each transducer, there is a trade-off between the depth of focus and the image resolution (contrast between the myocardial wall and the blood-filled chamber); as the depth of focus is increased, the image resolution decreases. Thus, there is an advantage to using the shortest focal length possible; however, this may not provide sufficient penetration to visualize a fetal heart in the maternal abdomen. There is a very broad range of depths in which a conceptus can be located in the pregnant rat and therefore a number of transducer heads of differing frequencies were empirically tested on different gestation days to determine which one was optimal over the experimental period.
Figure 2.1 shows images of GD 17 fetal hearts obtained using the RMV-704, RMV-712, RMV-707B, and RMV-710 transducers (respectively, Panels A – D). The RMV-704 transducer has the highest frequency (40 MHz) and therefore, provides the clearest image; however, its optimal resolution only reaches a depth of 6 mm. The RMV-710 transducer had the deepest penetration (15 mm); however, it emits at the lowest frequency (25 MHz) and has the lowest resolution. On GD 17 the RMV-712 transducer was optimal because the focal length of 9mm was sufficient to reach many, but not all, fetal hearts in utero and although the image obtained was not the sharpest, it offered sufficient contrast to visualize the structures. The RMV-707B transducer also provided sufficient resolution at a slightly greater depth than the RMV-712 transducer. Due to the wide range of depths at which conceptuses were located within the rat abdomen, it was evident that (1) there would always be implants above or below the plane of focus that could not be examined and (2) there was no scanning head that was universally applicable to all conceptuses on any given gestational day. In general, our experience was the RMV-707B and the RMV-712 scanning heads were most useful in the rat between GD 14 and GD 21.

It was also determined whether externalization of the fetuses would yield better image quality compared to in utero imaging. Recall that in theory the shorter focal length needed for an externalized fetus should permit the use of a transducer of higher frequency and resolution. Surprisingly, the images obtained from the fetuses in utero (Panel E) were superior to those collected when externalized (Panel F). There were also several other advantages of leaving the implants in situ. First, the externalized fetuses had fairly unstable heart rates (data not shown); this undermined the data integrity related to in
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Figure 2.1. Determining optimal conditions for ultrasound imaging of fetal rat hearts. Four scanheads of varying frequency and focal length (arrowhead) were used to obtain images of a control GD 17 fetal rat heart. The scanheads used were the RMV-704 (Panel A), the RMV-712 (Panel B), the RMV-707B (Panel C), and the RMV-710 (Panel D). The respective frequency and focal length is indicated for each scanhead. A comparison of B-mode heart images collected from a control GD 15 rat embryo in situ (Panel E) and externalized (Panel F); lv, left ventricle; rv, right ventricle; sep, septum; la, left atrium; ra, right atrium. These images were generated at Pfizer (Groton, CT).
uterine cardiac function. In addition, externalization precluded the long-term longitudinal assessment of cardiac development during gestation. Taken together, it was decided to conduct the remaining studies in utero.

The effects of isoflurane

The anaesthetic isoflurane is a reported teratogen; however, low-dose isoflurane is a recommended anaesthetic for high-throughput ultrasound studies (Stein et al., 2007). During preliminary exploratory assessment, we found that a high dose or long duration of exposure could have deleterious effects on cardiac function (data not shown). Generally, an appropriate anaesthetic plane could be maintained on 2 to 3% isoflurane, but in several instances 4 to 4.5% isoflurane was required. In these cases (three control dams, one treated dam) heart rates were highly unstable and the data was excluded. It was also noted that prolonged periods of anaesthesia resulted in decreased maternal and fetal heart rates; this occurred with equal frequency in control and DMO-treated dams (data not shown). For this reason we limited periods of anaesthesia to 45 min or less, which was sufficient time for preparation of the dam (removal of abdominal hair) and for four conceptuses to be located and fully interrogated with various high-resolution ultrasound modalities. During this period the maternal heart rates did not drop below 320 beats/min.

Identifying VSD with high-resolution ultrasound

One long-term goal of our laboratory is to track structural development of the heart longitudinally, in utero. We investigated whether DMO-induced structural anomalies of the heart, including VSDs, could be effectively identified using high-
resolution ultrasound. B-mode was used as an initial screen to localize hearts that appeared to have VSDs. Following ultrasound, fetal hearts were removed, fixed and sectioned to confirm the ultrasound observation. Figure 2.2 shows a B-mode image (Panel A) of a septal defect found in a fetal heart on GD 17, subsequently confirmed by histological sectioning (Panel B). It should be noted that in B-mode the open septum was more readily detected in real-time given that the user can visualize blood flowing across the top of the two ventricles; this cannot be done in a still. About a quarter of the fetuses could not be oriented in such a way as to permit the generation of predictive B-mode images. Under circumstances where satisfactory images were obtained, we had about 75% accuracy determining the patency of the septum using solely B-mode (data not shown).

Although histological sectioning allowed confirmation of a VSD, this method inhibits longitudinal assessment. Therefore, alternate ultrasound modalities to confirm ventricular septation defects in live fetuses in real-time were investigated. B-mode was still used to identify presumptive septal defects that would be subjected to further imaging. For illustrative purposes, Figure 2.2C and E show B-mode images of a GD 15 heart in two different orientations with the plane of focus on the exterior surface of the heart across the ventricles. The dashed line highlights the approximate location of the ventricular walls. Two corresponding M-mode images (Panels D and F) were taken in a location where the septum appeared to be present (Panel C) and a location where the septum appeared to be absent (Panel E). M-mode detected the ventricular walls in both orientations; however, the septum was only present in the image shown in Panel D, taken across the muscular region of the septum. The septum was absent from the M-mode
Figure 2.2. Confirmation of VSD in fetal rats using high-resolution ultrasound. Panel A depicts a B-mode image of a DMO-treated GD 17 heart. The arrow identifies the continuity between the left and right ventricle (lv and rv, respectively), indicating that the septum (sep) has not fused with the atrioventricular junction (avj). The fetus was harvested and the heart fixed and sectioned (Panel B) confirming the presence of a partially formed ventricular septum (sep) and a hole between the left and right ventricles (arrow). VSDs can also be confirmed using high-resolution ultrasound exclusively. Panels C and E are B-mode images of an untreated GD 15 heart in slightly different orientations (dashed line outlines the ventricles). The solid line marks the axes within Panels C and E from which M-mode images were obtained (respectively, panels D and F). The right and left ventricular walls (rvw and lvw, respectively) and septum are identified (sep). A septum is present in Panel D but absent in Panel F, indicating it is only partially formed on GD 15. To further confirm the breach between the left and right ventricle, Pulse-wave Doppler demonstrates the bi-directional rather than unidirectional flow (arrow heads; white shadow below 0 on the y axis). The RMV-712 transducer was used to obtain these images. These images were generated at Pfizer (Groton, CT).
image shown in Panel F, taken across the membranous region, indicating that fusion of the septum to the atrioventricular junction was incomplete. This was further confirmed using Pulse-wave Doppler over the location where the septum was suspected to be absent. As depicted in Panel G, a “shadow” appeared (arrow head) either above or below the major ejection fraction indicating there was bidirectional fluid flow in this region. No such “shadow” appears when the septum was closed. Using the above combination of B-mode, M-mode, and Pulse-wave Doppler, the patency of the septum was tracked in untreated fetuses on GD 14, 15, and 16, the days that span the window of ventricular septation. These data indicated that on GD 14 none of the hearts examined had a completely fused ventricular septum (Table 2.1), on GD 15 approximately half (54%) of the septa were closed, and by GD 16 all but one septum had closed (98%), suggesting there is a relatively broad window for this event. These same techniques were applied for the duration of this study to identify and confirm VSDs in DMO-exposed hearts. It should be noted that it was not always possible to obtain satisfactory images using all three ultrasound modes due to the orientation of the fetus in the uterus.

**Ultrasound can identify other structural defects of the heart**

In addition to the detection of VSDs, the high-resolution ultrasound biomicroscope was of sufficient resolution and sensitivity to detect both relatively common as well as rare and unexpected structural defects induced by *in utero* DMO exposure. Two incidences of possible bifurcated ventricular septum were first identified in B-mode in DMO-treated fetal hearts (Figure 2.3A; arrowhead and arrow). The presence of this anomaly was supported by an M-mode image taken across the ventricles.
The patency of ventricular septum was assessed in untreated dams on GD 14, 15, and 16, the period spanning the process of ventricular septal closure. B-mode was used to localize the ventricular septum within the heart. M-mode and Pulse-wave Doppler were used to respectively confirm whether the septum was complete and identify if there was blood flow between the left and right ventricles. This data was generated at Pfizer (Groton, CT).
Figure 2.3. Identification of various cardiac structural defects in fetal rats using high-resolution ultrasound. The presence of the bifurcated septum (arrowhead and arrow) in a DMO-treated GD 17 rat heart was first identified using B-mode (Panel A; lv, left ventricle; rv, right ventricle). The bifurcated septum was confirmed using M-mode (Panel B; arrowhead and arrow) and histological sections (Panel C; arrowhead and arrow). Common trunk was also identified in a DMO-treated GD 17 rat heart in B-mode (Panel D, arrow) and confirmed with histological sections (Panel E). A heart (GD 17) with a pericardial effusion (Panel F, arrowhead) was also identified in a DMO-treated rat fetus. The RMV-712 scanhead was used to obtain the ultrasound images in this figure. These images were generated at Pfizer (Groton, CT).
that revealed a signature double band indicative of two septa (Figure 2.3B). This rare defect was confirmed with histological sectioning (Figure 2.3C), and was identified in two separate DMO-treated litters. On GD 21, a DMO-treated heart with common trunk was identified using B-mode (Figure 2.3D) and confirmed with histological sectioning (Figure 2.3E). A DMO-treated heart with a pericardial effusion was also identified using B-mode (Figure 2.3F). Interestingly, the hearts that presented with these three anomalies (bifurcated septum, common trunk and the pericardial effusion) were also functionally compromised as evidenced by decreased ventricular contraction (M-mode) and heart rate (Pulse-wave Doppler; data not shown).

**Effects of dimethadione exposure on fetal heart function**

In light of the impaired cardiac function noted in some of the structurally compromised hearts, a final goal was to investigate if functional defects are more broadly associated with DMO exposure and/or DMO-induced defects. A representative control heart and an extremely adversely affected DMO-treated heart with prototypical structural and functional phenotypes are shown in Figure 2.4. Panels A and E depict, respectively, a phenotypically normal heart with a complete septum and a DMO-exposed heart with a VSD. Panel F highlights the “sponge-like” myocardial phenotype often noted in fetal hearts exposed to DMO in utero in contrast to the normal compacted myocardium in Panel B. The function of these hearts was investigated using Pulse-wave Doppler and M-mode of the high-resolution ultrasound. The DMO-exposed heart displayed reduced heart rate (Panel G) and weaker myocardial contractions (Panel H) than the control heart (Panels C and D). Based on these observations, we embarked on a larger multi-litter
**Figure 2.4.** Comparison of structural and functional properties of a DMO-treated and a control fetal rat heart. A histological section of an untreated normal GD 21 heart (Panel A) and a DMO-treated GD 21 heart with a VSD (Panel E; arrowhead). Higher magnification of the inset reveals normal myocardial density in the untreated heart (Panel B) and a “sponge-like” myocardium in the heart exposed to DMO (Panel F). Pulse-wave Doppler demonstrates reduced heart rate (number of peaks over unit time; x-axis) and ejection velocity (peak height; y-axis) in the DMO-treated heart (Panel G) when compared to the control heart (Panel C). M-mode imaging in a plane crossing the septum (sep) illustrates reduced contractility (arrow length) of the left and right ventricular walls (lvw and rvw, respectively) in the DMO-treated heart (Panel H) when compared to control (Panel D). Tracings in (Panels D, H) outline the ventricular walls in diastole (dia; solid arrow) and systole (sys; dashed arrow). The RMV-712 scanhead was used. These images were generated at Pfizer (Groton, CT).
campaign to further investigate the effect of *in utero* DMO exposure on heart rhythm and contractility.

*Effects of dimethadione exposure on fetal heart rhythm*

Figure 2.5A depicts a standard Pulse-wave Doppler scan used to extrapolate the heart rate and rhythm of each fetus. A scatter plot of these data (Panel B) demonstrates a high degree of variability and a statistically significant rise in heart rate of control fetuses on GD 21 when compared to GD 14, 16, and 17. Furthermore, this plot illustrates that with the exception of GD 16, the average heart rate of the DMO-treated group was significantly reduced compared to the control group.

To further clarify the decrease in mean heart rate in DMO-exposed fetuses, we assessed the incidence of bradycardia, which was defined as a heart rate 10% below the mean heart rate of the control group on the same gestational day (compare Figure 2.6A vs. B). The overall incidence of bradycardia in the DMO-treated group was 45%, twice that of the control group (23%). The high incidence of bradycardia in the controls may reflect our broad definition of bradycardia (10% less than the mean control heart rate) and therefore may not be of pathologic significance; however, these data suggest that the decrease in heart rate was reflected, at least in part, by the increased incidence of bradycardia in DMO-treated fetuses (Figure 2.6C; Fisher’s exact test, p = 0.0001).

A significantly greater incidence of dysrhythmia was also noted in DMO-treated fetuses compared with controls. A dysrhythmia was defined as a heart rate with a standard deviation greater than 5% of the mean. Representative Pulse-wave Doppler
Figure 2.5. Assessment of mean heart rates of the DMO-treated and the control fetal rats. Embryo/fetal heart rate is significantly reduced after DMO treatment. Pulse-wave Doppler tracings (Panel A) were used to calculate the mean heart rate (beats per minute) using a peak-to-peak methodology (arrowhead to arrowhead). The heart rates of each control (solid circle) and DMO-treated (open square) embryo imaged on GD 14, 16, 17, and 21 are depicted on a scatter-plot in Panel B. The mean heart rate ± standard deviation is depicted for each group on each gestational day and the mean value is listed below each plot (x). Numbers in parentheses are the number of fetuses imaged. * statistically significant difference between control and DMO-treatment; ^ statistically significant difference compared to GD 14, 16, and 17 (two-way ANOVA; p=0.0014 using Tukey post hoc test).
Figure 2.6. Incidence of bradycardia in the DMO-treated and control fetal rats. Panels A and B are respective Pulse-wave Doppler scans of a control versus a DMO-treated heart. Fetal heart rates were extrapolated from Pulse-wave Doppler scans by determining the peak-to-peak distance (line between arrowheads). The interbeat interval is longer in the DMO-treated heart (solid and dashed line on Panel B) compared to the control heart (solid line on Panel A). Panel C shows the absolute incidence of bradycardia in control and DMO-treated fetuses on GD 14, 16, 17 and 21. Parentheses are the number of litters used. Total incidence is the cumulative percent incidence per treatment group across all gestation days. The incidence of bradycardia was significantly increased after in utero exposure to DMO (Fisher’s Exact Test, p = 0.0001).
tracings illustrate the variability in heart rate of a DMO-exposed fetus (Figure 2.7B) compared with the relatively constant heart rate of the control fetus (Figure 2.7A). In the control group, there was a single incidence of irregular heart rhythm occurring on GD 14. In the DMO-treated group, there were eight cases of dysrhythmia (3 on GD 14, 2 on GD 16, 2 on GD 17, and 1 on GD 21) resulting in significantly increased total incidence of 11% (Fisher’s Exact Test, p = 0.0135). Two fetuses on GD 16 and two fetuses on GD 17 with dysrhythmia also had a VSD. The GD 21 fetus with dysrhythmia had a complete septum.

**Effects of dimethadione exposure on fetal heart contractility**

Initially, contractility of the hearts was assessed by measuring the height of each peak in Pulse-wave Doppler mode, indicative of the ejection velocity of the blood through the outflow tract during ventricular contraction. It was found that the ejection velocity was markedly decreased in some DMO-treated fetuses compared to control (Figure 2.4 compare peak height in panels C and G); however, attempts to measure this parameter reproducibly proved difficult. The orientation of the outflow tract relative to the transducer probe is critical and slight deviations from 0° resulted in significant changes in peak height; consequently these values were highly variable and not statistically significant (data not shown).

As an alternative, M-mode tracings were analyzed to quantitively measure contractility at different stages of gestation. The Visualsonics™ software was used to trace the contracting ventricular walls and calculate the mean stroke volume, ejection fraction, fractional shortening, and cardiac output (Table 2.2). Two-way ANOVA
Figure 2.7. Incidence of dysrhythmia in the DMO-treated and control fetal rats. Panels A and B show Pulse-wave Doppler scans with the beat-to-beat heart rates on GD 14 for a control heart and a DMO-treated heart, respectively. Note the beat-to-beat variability in each panel. Panel C shows the absolute incidence of dysrhythmias in control and DMO-treated fetuses on GD 14, 16, 17 and 21. Parentheses are the number of litters examined. The cumulative percent incidence of dysrhythmia was significantly increased after in utero exposure to DMO (Fisher’s Exact Test, p = 0.0135).
B-mode was used to localize and orient the heart after which M-mode images were obtained. Using the software tools supplied with the Vevo system, the contracting ventricular walls on the M-mode image were traced and stroke volume, ejection fraction, fractional shortening, and cardiac output were calculated from the resulting trace data. The average of these values for each group was determined and two-way ANOVA was performed with Tukey post hoc tests to determine statistically significant differences (p < 0.05) across gestational day and between control and the DMO-treated fetuses. The variable fetal numbers were the result of an inability to place all fetuses in the correct orientation for M-mode imaging. Gestational effect: aSignificance from GD14; bSignificance from GD16; cSignificance from GD17. Treatment effect: *Significance between control and DMO-treated on the same gestational day; †Treatment effect is approaching significance (0.05 < p < 0.06).

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Table 2.2. Assessment of cardiac contractility during gestation
revealed that both gestational and treatment effects were evident. For stroke volume and cardiac output, GD 21 values were significantly increased relative to all other gestational days. For ejection fraction, GD 21 values were significantly increased relative to GD 14 and GD 16. DMO treatment also significantly altered cardiac performance on specific gestational days. There was a trend toward a decreased stroke volume after DMO treatment on GD 17, but it only reached statistically significant levels on GD 21. DMO exposure significantly decreased the ejection fraction on GD 17 and GD 21. Fractional shortening was significantly decreased after DMO-treatment on GD 17 and not quite statistically significant on GD 21 (0.05 < p < 0.06). Cardiac output was significantly impaired in DMO-treated fetuses only on GD 21.

**DISCUSSION**

Trimethadione is a potent inducer of heart malformations in humans and a number of experimental animal models (Feldman *et al.*, 1977; Rischbieth, 1979; Veuthey *et al.*, 1990; Solomon *et al.*, 1997; Fleeman *et al.*, 2004). It is believed dimethadione, the N-demethylated metabolite of trimethadione, is the proximate teratogen (Buttar *et al.*, 1978; Wells *et al.*, 1989) and we have previously demonstrated direct administration of DMO induces a number of cardiac malformations including both membranous and muscular ventricular septation defects, outflow tract anomalies, and “sponge-like” myocardium, which would be anticipated to impair cardiac function both *in utero* and postnatally (Weston *et al.*, 2011). This prompted our interest in determining the feasibility of using high-resolution ultrasound to identify and follow structural malformations longitudinally during gestation and to assess their impact on *in utero* cardiac function in the rat.
conceptus. The results of the current study lead us to conclude that, as previously shown in the mouse (Spurney et al., 2004), ultrasound is a valuable non-invasive tool for the detection of heart malformations and functional defects in the fetal rat following in utero treatment with a known cardiac teratogen. The DMO-induced dysrhythmia, heart rate suppression, and reduced contractility reaffirm the strong link between structure and function in the developing fetal heart. Moreover, if the current findings for DMO are more broadly applicable to other chemicals that induce congenital heart defects, it suggests functional deficits may be under-reported and of significant concern.

Although ultrasound has been used clinically for decades to assess in utero cardiac structure and function, its utility in small laboratory animals has been limited by its relative lack of resolution. With the advent of high-frequency ultrasound biomicroscopes in the past decade (Foster et al., 2002; Zhou et al., 2002), the assessment of in utero cardiac structure and function has become possible in the mouse and rat embryos (Bjornerheim et al., 2001; Leatherbury et al., 2003). There is a trade-off between high-frequency (high-resolution) and tissue penetration. We therefore tested four scanning heads of varying frequencies with the goal of identifying the optimal transducer head for use on specific gestational days. Unfortunately, each litter had a cohort of conceptuses either above or below the plane of focus and unavailable for assessment, and thus there was no “ideal” scanning head for any particular gestational day. Instead, the RMV-707B and the RMV-712 appeared to have sufficient resolution and tissue penetration to be useful for the assessment of a subgroup of embryos within any litter between GD 14 and 21. The inability to assess all rat conceptuses within a litter has been similarly reported by other investigators who required 20 dams to obtain 45 embryo/fetal
assessments (Ypsilantis et al., 2009). Moreover, due to the motility of the uterus, different conceptuses were within the focal plane on different gestational days and it was difficult to identify, with certainty, fetuses from day to day within the same dam, unless they were immediately adjacent to the cervix. This points to a challenge of this technology because a truly longitudinal assessment of the same fetus across gestation is, in fact, very difficult. These same issues have been similarly reported in mice (Zhou et al., 2002). Some researchers exteriorize the conceptuses for their study in order to have greater control over the orientation of every pup in the litter (Zhou et al., 2002). This procedure was not employed in the current study (except during the exploratory phase) for several reasons. First, in contrast to the findings of Zhou et al., (2002) this procedure did not improve image quality in our hands. Second, exteriorization induced fetal, but not maternal heart rate instability, although it is not clear whether changes in the anaesthetic protocol may counter this instability. Although the previously outlined technical challenges prevented us from tracking a specific fetus longitudinally across gestation, leaving it in the uterus in situ allowed sampling of about one third of a litter at several time points during gestation, thereby potentially reducing the number of animals required for study conduct.

In this study, the duration of anaesthesia was limited to 45 minutes or less to avoid changes in both maternal and embryo/fetal heart rate. As a result we were limited to examining four conceptuses per litter. The effect of isoflurane on cardiac performance remains controversial with opposite effects being reported by different investigators. For example, a study investigating the effects of anaesthesia on echocardiographic assessment found isoflurane had negative inotropic and chronotropic effects in rats (Stein
et al., 2007), whereas another study found isoflurane had no significant effect on heart rate or cardiac performance in chicks (Wojtczak, 2000). Anecdotally our experience was that by increasing the maintenance dose of isoflurane from 3% to 4.5% there was a dramatic increase in the incidence of dysrhythmia in the fetuses of three dams that were refractory to our standard anaesthetic maintenance protocol (2% to 3% isoflurane).

Although high-resolution ultrasound has been touted as a “high-throughput” approach for the identification of malformations in an N-ethyl-N-nitrosurea-induced malformation screen in mice (Yu et al., 2004), in our hands this technology was slower than standard fresh tissue examination; however, its non-invasiveness was a decided advantage for both Yu et al., (2004) and this study. In our experience, B-mode alone was accurate about 75% of the time in the assessment of patency of the ventricular septum. The use of M-mode and Pulse-wave Doppler as an adjunct to confirm B-mode findings resulted in 100% accuracy; however, due to the embryo/fetal orientation within the abdomen it was not always possible to provide confirmation. Nevertheless, when the heart is in the correct orientation high-resolution ultrasound was non-invasive and provided sufficient data for the accurate assessment of ventricular septum development. Using all three modalities, B-mode, M-mode, and Pulse-wave Doppler, the closure of the ventricular septum was tracked on GD 14, 15, and 16 with respective rates of completed septum fusion being 0, 54 and 98%. This reflects previous reports in which septation in the rat is initiated around GD 13 and completed by GD 16 (Sissman, 1970; DeSesso, 1997; Laffin et al., 2004). It is unclear whether the single unclosed septum we noted in a control fetus on GD 16 was within the range of “normal”, a developmental delay, or representative of a spontaneously occurring VSD.
Unexpectedly, high-resolution ultrasound identified several rare and novel DMO-induced malformations that we did not previously detect when using standard fresh tissue examinations (Weston et al., 2011). These included two instances of bifurcated septum in two separate DMO-treated litters and pericardial effusion. The bifurcated septum and pericardial effusion would have been detectible with thorough histological sectioning but this approach is prohibitively time consuming and necessitates sacrificing the litter. Further analysis with other ultrasound modes determined that cardiac performance was compromised in these abnormal hearts, as evidenced by decreased heart rate and ventricular contraction. It is important to note that although the in utero cardiac function was compromised in the bifurcated hearts, their long-term significance on cardiac function is unknown, in part, since it is unclear whether they persist postnatally.

We recently reported significant structural and histological anomalies in hearts exposed to DMO, including hypoplastic atrium, muscular and membranous ventricular septation defects, and “sponge-like” myocardium (Weston et al., 2011), all of which might be expected to impair cardiac function. These observations, taken together with reports of the interrelationship between structure and function in the embryonic heart and our preliminary observations of compromised function in hearts with severe defects prompted us to hypothesize that hearts exposed to DMO in utero are also functionally impaired. Indeed a number of parameters were adversely affected by DMO treatment, including an 11% incidence of dysrhythmia and a 45% incidence of bradycardia in DMO-exposed conceptuses. Although not every DMO-exposed fetus developed bradycardia, the effect caused a significant reduction in average heart rate in the DMO-treated groups on GD 14, 17, and 21. Only on GD 16, was there no difference between
the average heart rate of the DMO-treated and the control groups and we speculate that
this may be the result of the high variability in heart rate in both the DMO-treated and
control groups. We attribute this variability, in part, to the fact that some fetuses had
heart malformations while others did not, and the possibility that on a given gestational
day fetuses may exhibit a wide range of developmental landmarks reflecting a diversity
of “developmental ages”. It is unclear whether the decrease in heart rate is a direct or
indirect effect of chemical exposure because DMO is reported to be a potent HERG
channel blocker in the mouse embryonic heart (Azarbayjani & Danielsson, 2002). On GD
14, the earliest day we monitored heart rate, the maternal serum levels of DMO were
between 50-150 µg/mL (Ozolinš, manuscript in preparation); however in mouse,
maternal blood concentrations of 1.94 mM (250 µg/mL) did not induce bradycardia or
dysrhythmia whereas 3.2 mM (420 µg/mL) did. Thus, unless HERG in embryonic rat is
more sensitive to DMO inhibition than mouse embryonic HERG, this suggests the
bradycardia and dysrhythmia noted in the current study is not due to direct HERG
channel inhibition but likely an indirect, long-term or possibly permanent effect,
independent of HERG blockade.

The embryo/fetal heart rates reported in the current study differ from previous
reports. For example, the respective GD 14 and 16 heart rates for control rats in the
current study were 172 ± 23.0 and 166 ± 25.2 beats/minute, whereas Ypsilantis et al.,
(2009) reported 194 ± 13 and 224 ± 28 beats/minute. The cause of this discrepancy is
unclear but may be related to strain differences; Sprague-Dawley was used in the present
study whereas the Wistar was used in the Ypsilantis study (2009). There were also
differences in the anaesthetic protocol used in each study. The rats in the Ypsilantis study
were administered 2% sevofluorane and were cumulatively exposed to four or five sessions of anaesthesia. In contrast, the rats in the present study were administered 2-3% isoflurane and only exposed to a maximum of two sessions of ultrasound, between two to seven days apart. The differing cumulative exposures to anaesthetic might explain the differing heart rates in each study.

We also found that DMO treatment had a negative inotropic effect on fetal hearts leading to a reduced ejection fraction and cardiac output compared to control. There was also a trend toward reduced fractional shortening and reduced stroke volume in the DMO-treated group compared to the control group; however, this did not quite reach the level of statistical significance. We believe this trend is biologically significant and may be partially masked by the high variability which may be due, in part, to the presence of both structurally normal and abnormal hearts. Decreased contractility of the DMO-exposed hearts may have been due, in part, to the “sponge-like” myocardial phenotype that was noted in histological sections of some hearts. This phenotype has been observed clinically (Budde et al., 2007; Dogan and Demircin, 2008) and often presents with other congenital cardiac malformations, including VSD (Dogan and Demircin, 2008), suggesting there may be a mechanistic link. Ejection velocity was also highly variable and we attribute this to the fact that Pulse-wave Doppler must be conducted with the transducer head at a 0° angle relative to the outflow tract. Subtle changes in this angle results in highly variable peak heights.

Finally, it was of interest that the four dysrhythmic hearts found on GD 16 and 17 also presented with VSD, suggesting that structural defects in the fetal heart may also compromise seemingly unrelated functional parameters. Unfortunately, the design of the
current study did not allow us to go back retrospectively and link specific structural defects with specific deficits in cardiac performance across a broader cohort of fetuses. This was partially related to the fact that within the uterus it was often difficult to obtain simultaneous assessment of structure and function in all hearts. This limitation of *in utero* ultrasonography suggests that the ability to alter the fetal orientation may be a decided advantage of exteriorization. Studies are underway in our facility to assess the link between structural and functional deficits more directly.

More disturbing was the observation that the dysrhythmic heart on GD 21 had an intact septum. This suggests heart teratogens may cause significant functional deficits in the absence of visible structural malformations and these defects may continue postnatally. Studies are also underway to investigate the long-term effects of *in utero* DMO-treatment.

In conclusion, high-resolution ultrasound is a unique research tool that, despite some minor limitations, facilitates the non-invasive assessment of *in utero* rat cardiac structure and function. In this study, high-resolution ultrasound identified several novel DMO-induced heart malformations, previously unreported using standard fresh tissue examinations. Moreover, these studies demonstrate that exposure to dimethadione, and likely other heart teratogens, may result in significant functional deficits including dysrhythmia, bradycardia and reduced cardiac output. Together, this underscores the link between structure and functions and suggests that ventricular septal defects may be a sentinel for serious functional anomalies.
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CHAPTER 3

POSTNATAL ALTERATIONS IN RAT HEART STRUCTURE AND FUNCTION AFTER IN UTERO EXPOSURE TO DIMETHADIONE, THE N-DEMETHYLATED METABOLITE OF THE ANTICONVULSANT TRIMETHADIONE

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ABSTRACT

BACKGROUND: We previously showed that dosing pregnant rats with dimethadione (DMO), the N-demethylated teratogenic metabolite of the anticonvulsant trimethadione, induces a high incidence of ventricular septal defects (Weston et al., 2011) as well as in utero functional deficits including an increased incidence of bradycardia and dysrhythmia and a reduction in cardiac output and ejection fraction in embryos up to 10 days following the final dose (Purssell et al., 2012). We hypothesized that these functional deficits will persist postnatally. METHODS: Pregnant Sprague-Dawley rats were administered six 300mg/kg doses of DMO every 12 hours beginning on the evening of gestational day 8. Dams were allowed to reach parturition and postnatal cardiac function was assessed in a cohort of surviving pups using telemetry, high-resolution ultrasound, and electrocardiography (ECG). RESULTS: Adult rats exposed to DMO in utero had an increased incidence of irregular heart rhythm at high levels of activity as well as under anaesthesia, which ECG suggested was due to disturbances in the electrical conduction in the heart. DMO-exposed rats also had elevated mean arterial pressure and cardiac output, a larger left ventricular volume during systole, and greater physical activity within the cage compared to controls. Importantly, many DMO-exposed rats had functional deficits in the absence of a persisting structural defect. CONCLUSIONS: Exposure to DMO in utero causes deficits in the cardiac conduction system that persist into postnatal life in the rat, despite the absence of visible structural anomalies. We speculate this is not unique to DMO, indicating there may be health implications for infants with unrecognized gestational chemical exposures.
INTRODUCTION

A ventricular septal defect (VSD) is a breach in the septum separating the left and right ventricles that allows oxygenated and deoxygenated blood to mix. VSDs are not only the most common congenital heart defect, but also the most common birth defect overall (Rosamond et al., 2008; Bernier et al., 2010; Dolk et al., 2011). Approximately 85 - 90% of clinical VSDs spontaneously resolve within the first year of life and are believed to not require any further clinical intervention (Roguin et al., 1995; Du et al., 1998); however, unresolved VSD may require surgical repair depending upon location and severity (Hoffman and Kaplan, 2002; Asou, 2011). The presence of an uncorrected VSD can lead to pulmonary arterial hypertension as a result of hemodynamic changes due to the breach between the left and right ventricles. If the pressure of the pulmonary circulation reaches that of the systemic circulation, the shunt between the ventricles can reverse and allow deoxygenated blood to enter the left ventricle and cause hypoxia, a condition known as Eisenmenger’s syndrome (Duffels et al., 2007; Engelfriet et al., 2007).

It was believed that the surgical repair of the structural defect would resolve any associated functional deficiencies; however, clinical evidence suggests this may not be the case as patients with a surgically repaired VSD may still develop pulmonary arterial hypertension. Furthermore, these patients are at a greater risk for the development of conduction defects and cardiomyopathies (Kidd et al., 1993; Pacileo et al., 1998; McLaughlin et al., 2004; Roos-Hesselink et al., 2004; van der Velde et al., 2005). This suggests that individuals born with a structural defect may also have inherent deficits not
readily detected at parturition but that predispose them to developing more severe functional pathologies as they age.

The co-occurrence of both structural and functional defects is to be expected when one considers genetic mutations that are associated with both congenital heart defects and functional pathologies. For example, three major transcription factors, T-box transcription factor 5 (Tbx5), homeodomain-containing transcription factor 2.5 (Nkx2.5), and the zinc finger transcription factor 4 (GATA4) have been implicated in the clinical incidence of VSD (Basson et al., 1997; Li et al., 1997; Schott et al., 1998; Benson et al., 1999; Garg et al., 2003). These transcription factors also control postnatal cardiac function as they co-regulate the expression of numerous genes that have critical roles in cardiac conduction and contractility. Tbx5 regulates sarcoendoplasmic reticulum calcium ATPase isoform 2a (SERCA2a) (Zhu et al., 2008), a calcium pump that is critical to the process of diastolic relaxation (MacLennan and Kranias, 2003). Nkx2.5 regulates the expression of minK (Jay et al., 2004; Pashmforoush et al., 2004), a protein component of potassium channels involved in cardiac repolarization (Li et al., 1996; Sanguinetti et al., 1996; McDonald et al., 1997). Tbx5 and Nkx2.5 co-regulate cx40 (Bruneau et al., 2001), a gap junction protein involved in electrical coupling between myocytes (Kumar and Gilulu, 1996), and myosin light chain (MLC2v) (Lyons et al., 1995; Tanaka et al., 1999; Ghosh et al., 2001), a protein component of the myocardial wall. All three transcription factors regulate natriuretic propeptide A (NPPA) (Takeuchi et al., 2003), a peptide hormone involved in fluid and electrolyte balance and blood pressure regulation (Needleman et al., 1985). Postnatal cardiac functional pathologies characteristic of individuals with mutations in these genes resemble those of individuals born with VSD of
unknown etiology, suggesting that the observed functional pathologies may be due to a disturbance in gene expression programs during development.

While 10% of the incidence of VSD is due to genetic mutations (Hoffman, 1990), the remaining 90% has been attributed to environmental factors which include chemical exposures; therefore, using in utero chemical exposures as a tool to study VSD and associated functional anomalies is a clinically relevant approach. We have chosen to use dimethadione (DMO), the teratogenic metabolite of the antiseizure medication trimethadione (TMD) (Butler et al., 1952) for several reasons. Firstly, the parent compound, TMD was removed from the pharmaceutical market because of its potent ability to induce VSD and other birth defects in infants (Zackai et al., 1975; Feldman et al., 1977; Rischbieth, 1979), indicating that the results of the study of DMO should be clinically translatable. Secondly, we have demonstrated the utility of using DMO to generate an approximately 74% incidence of VSD in the absence of significant maternal or embryo/fetal toxicity (Weston et al., 2011). Thirdly, DMO treatment induces both membranous and muscular VSD, representative of the clinical variability in localization of VSD (Weston et al., 2011). Lastly, in utero exposure to TMD in rats produces offspring in which approximately 80% of VSD close spontaneously postnatally (Fleeman et al., 2004), reflecting the clinical rate of postnatal VSD closure (Hoffman and Kaplan, 2002).

Recently, we demonstrated that DMO exposure during a critical window of heart development induced a high incidence of structural anomalies at parturition (Weston et al., 2011) as well as deficits in fetal rat heart function in utero (Purssell et al., 2012). Using high-resolution ultrasound, it was apparent that DMO-exposed fetuses had a delay
in closure of the interventricular septum, in addition to a reduction in cardiac output and heart rate, as well as a greater incidence of bradycardia and dysrhythmia (Purssell et al., 2012). These functional abnormalities persisted in fetuses up to 10 days after the final dose of DMO, suggesting its deleterious effects are long term and possibly permanent. This has prompted us to undertake the current study to determine whether the pathophysiological changes induced by in utero DMO exposure persist into adulthood.

MATERIALS AND METHODS

Animals

Time-mated Sprague-Dawley rats [Crl:CD(SD)] were obtained from Charles River Inc. (St-Constant, QC). The morning after copulation was designated as GD 0. Dams were housed individually and the pups were born at Queen’s University. After weaning, the offspring were housed with littermates and after surgery they were housed individually. All rats were housed in polypropylene shoebox cages with heat-treated hard wood chip contact bedding (Beta chips®, Northeastern Products Corp. Warrensburg, NY). Room conditions included a minimum of 12 air changes per hour with air filtered through at least 90% - 95% efficiency filters and then through HEPA filters, relative humidity of 20 – 50% depending on the season, temperature of 70 ± 5°F, and a 12 hour light/dark cycle (lights on/off 07h00/19h00). Certified Rodent Diet 5001 (PMI® Nutrition International, LLC, Richmond, IN) and drinking water from a municipal source and further purified by reverse osmosis were provided ad libitum. All procedures underwent veterinary review and were approved by the Queen’s University Animal Care Committee.
**Chemicals**

Dimethadione was purchased from Sigma Aldrich Inc. (St. Louis, MO). Isoflurane was purchased from Pharmaceutical Partners of Canada Inc. (Richmond Hill, ON).

**Treatment**

The day prior to the initiation of dosing, dams were separated into control (CTL) and DMO-treated groups using body weight stratification to ensure the mean body weights of each group were as closely matched as possible. A total of six 300mg/kg doses of DMO (60 mg/mL drug solution) were administered to the dams by oral gavage (5mL/kg dose volume) every 12 hours, beginning at 19h00 on GD 8. Control dams received an equivalent volume of distilled water. This dosing regimen was used as it has been shown to induce a 74% incidence of VSD (Weston et al., 2011) as well as deficits in cardiac function *in utero* (Purssell et al., 2012). Six DMO-treated offspring from three litters survived and eight control offspring were selected from two litters to be used in postnatal assessment. These rats were labelled CTL1 – 8 and DMO1 – 6, respectively.

**Postnatal Assessment of Cardiovascular Function**

The study design is outlined in Figure 3.1. Following dosing of the pregnant rats and the initial stratification of the newborn pups, the pups were left alone to mature. Assessment of cardiovascular function began at approximately eight weeks by implanting telemeters for the assessment of cardiovascular function under basal conditions.
Figure 3.1. Study design used in Chapter 3. Rats were dosed six times with either 300mg/kg DMO or water for controls between GD 8 and GD 11. The original goal was to stratify the pups following parturition according to VSD status; however, low pup viability and the unavailability of ultrasound scanning heads with an appropriate focal length prevented this approach. Surviving pups were implanted with telemeters at approximately eight weeks of age. Following two weeks of postoperative recovery, the telemeters were activated for one month to assess cardiovascular parameters including heart rate and rhythm, blood pressure, and activity at basal conditions. At approximately one year of age, high-resolution ultrasound coupled with electrocardiography was used to assess electrical conduction, heart rhythm, dimensions, and contractility. At this stage, the patency of the septum was assessed to allow for stratification based on the presence or absence of persisting VSD.
Telemetry was used for data collection because it allowed assessment of cardiovascular function in free-moving unrestrained rats. The telemeters were active for a period of 32 days. Telemetry data included continuous assessment of heart rate and rhythm, blood pressure, and activity of each rat. Between ten months and one year of age, each rat underwent one session of ultrasound examination of structural and functional parameters of the heart including heart dimensions and measurements of contractility, as was done previously in fetal rats (Purssell et al., 2012). Furthermore, ultrasound allowed for the assessment of persisting VSD. The ultrasound system incorporates electrocardiography (ECG) and so allowed us to not only assess heart rhythm, but also the electrical conduction through each heart.

Stratification

As indicated in Figure 3.1, our goal was to stratify the rat pups into four groups at four weeks of age based upon the presence or absence of VSD: (1) Control, no VSD, (2) DMO-treated without VSD, (3) DMO-treated with a VSD at birth that spontaneously resolved by weaning, and (4) DMO-treated with a VSD at birth that remains open. The objective was to assess the severity of postnatal functional deficiencies under differing conditions of VSD resolution. High-resolution ultrasound was to be used to assess the integrity of the septum within 24 hours of parturition to determine the initial presence of a VSD, and again at four weeks to determine if the VSD resolved. Due to unexpectedly high pup mortality (explained in full in the results section) and the unavailability of the appropriate ultrasound probe, we were unable to assess VSD status immediately after birth. Instead the integrity of the interventricular septum was assessed in adult rats at the
termination of the experiment using high-resolution ultrasound (indicated in italics on Figure 3.1). The three groups were: (1) Control, (2) DMO-treated without VSD at one year of age, and (3) DMO-treated with a persisting VSD at one year of age.

Telemetry Methods and Surgery

Telemetry involves the surgical implantation of a telemeter into the abdominal cavity to assess cardiovascular function in the conscious adult rat. It is the most accurate method of assessing physiological parameters as the animal is neither handled, nor exposed to anaesthetics. The telemetry system used was from Data Sciences International™ (St. Paul, MN). Rats were allowed to reach 250g (approximately 8 weeks for females) before undergoing surgical implantation. Anaesthesia was induced by administering 5% inhaled isoflurane gas and maintained with 2 to 3% inhaled isoflurane gas in 100% oxygen at 1L/min flow rate through a nose cone. Subcutaneous injections were given of Metacam (2mg/kg) for analgesia and lactated ringers solution (10mL) for hydration. The rat was positioned supine and the paws were taped down to the surgical table. The abdomen of the rat was shaved from the subxiphoid space to the pelvis and washed with Betadine scrub, iodine, and isopropyl alcohol. A 4 to 5 cm incision was made down the midline of the abdomen of the rat and the abdominal aorta was exposed using sterile cotton-tipped applicators to gently move aside the intestines and other vital organs. The abdominal cavity was lined bilaterally with 4 x 4 gauze pads soaked in sterile saline and retractors were used to gently widen the cavity and expose the abdominal aorta. A 3mm portion of the abdominal aorta was teased from the surrounding fat and muscle using cotton-tipped applicators and the aorta was clamped just below the renal
artery and vein. The aorta was punctured using a 26-gauge needle bent at a 90° angle from the bevel and the thin-walled section of the transmitter tubing was inserted through the puncture site. Once in place, the area was dried, covered with a 2 mm by 2 mm square of hernia mesh and 2 drops of Vetbond were applied to the point where the catheter entered the aorta. The body of the transmitter was positioned on top of the intestines running lengthwise within the intraperitoneal cavity. The muscle layer of the abdomen was stitched back together using interrupted stitches with non-absorbable suture (5-0 or 6-0), three of which were passed through the casing of the transmitter body to adhere it to the abdominal wall. The epidural layer was closed with 3-0 monocryl using internal interrupted stitches. The outside of the incision was washed with sterile saline. Delivery of the isoflurane was stopped and the rat was monitored until the righting reflex had returned and the respiratory rate stabilized. The rat was given food supplements (recovery gel), fluids (5 – 10mL lactated ringer solution, subcutaneously), and an analgesic (Metacam, 1mg/kg, subcutaneously every 24 hours) for a minimum of 3 days following surgery. All the rats were monitored daily and their weights recorded biweekly. The rats were allowed two weeks to recover from the surgery before the telemeters were activated so as to prevent confounding of the cardiovascular data by postoperative stress. Following surgery, it was determined that the telemeter in DMO4 was non-functional, resulting in the collection of telemetry data for only five DMO-treated rats.

**Telemetry Data Analysis**

The telemeters record the heart rate (beats/min), the arterial blood pressure (mmHg), and the physical activity (counts) of the rats. Activity counts are an estimate of
the movement of the rat in the cage. The signal between the receiver and the telemeter changes with the location and the orientation of the rat. When the signal changes, the telemeter generates pulses that are converted into standardized units of activity (Clement et al., 1989). When the rat is not moving (ie. sleeping), the activity count is 0. The average activity count for a rat that is awake is approximately 3 (Molčan et al., 2009). The activity count scale does not have an upper limit.

The telemeters were set up to collect 30 seconds of continuous data at 12 minute intervals. The averages and standard deviations of each parameter were calculated for each 30 second recording period. Furthermore, the daily average and standard deviation of each parameter were calculated for each rat.

Daily averages of the heart rate, mean arterial pressure (MAP), and activity were analyzed by two-way analysis of variance (ANOVA) to determine if there were statistically significant differences between the control and DMO-treated rats. Due to differences in activity levels, the average heart rate and MAP were stratified by activity level and analyzed.

An analysis of the continuous beat-to-beat data was conducted in order to investigate the heart rhythm of each rat. Due to the enormity of the telemetry data set, three time points were selected per day for analysis: 00h00, 06h00, 18h00. These time points were chosen as they encompass various points in the circadian cycle and they are times in which no animal care personnel were present. The average heart rate and MAP were stratified by these three time points and a two-way ANOVA was conducted to determine if the time of day affected these parameters for each treatment group.
The beat-to-beat data was qualitatively assessed using Poincaré plots. Poincaré plots illustrate the variability in the heart rate by plotting each interbeat interval (the time between two heart beats) against the following interbeat interval. The spread of the data points on the plot represent the variability in the heart rate. This variability can be either physiological (heart rate increasing or decreasing in a normal manner) indicated by when the slope of the best-fit line through the data points approximates one, or pathological (irregular heart rhythm) indicated when there is no pattern to the spread of the data points. Poincaré plots were constructed for time points in which the rat had a high level of activity.

The beat-to-beat data was quantitatively analyzed by calculating the coefficient of variation for the heart rate (standard deviation divided by the average heart rate for 30 seconds of data) at each time point. A t-test was used to determine if there was a significant difference between the coefficients of variation between the control and DMO-treated groups. Furthermore, the beat-to-beat heart rate data for each time point was classified as having a normal level of variability or being hypervariable. The heart rate at any given time point was defined as hypervariable when the standard deviation was greater than 5% of the average heart rate (coefficient of variation > 0.05) (Purssell et al., 2012). A Fischer’s Exact Test was used to determine if there was a difference in the incidence of hypervariability between the control and DMO-treated group.

**Ultrasound Procedure**

The high-frequency ultrasound imaging system used was the Vevo770 (VisualSonics Inc., Toronto, ON). Ultrasound scanning was done in collaboration with
Kristiina Aasa. The rats were anaesthetised using a VisualSonics™ isoflurane vaporiser (VisualSonics Inc., Toronto, ON). Anaesthesia was induced as previously described. The rat was positioned supine on a heating pad and the paws were taped down to the ECG pads. The temperature of the rat was maintained at 37°C using a heat lamp and the heart rate of the rat was monitored and maintained at a minimum of 320 beats/min through adjustment of anaesthesia. Chest hair was removed using depilatory cream (Nair®, Church and Dwight Co. Inc, Princeton NJ). Pre-warmed ultrasonic gel was layered over the location of the heart on the rat for in situ imaging.

Three different modes of the ultrasound were used for assessment of the adult rat hearts. Brightness mode (B-mode) produces a two-dimensional (2D) image of the heart by aligning scanlines along the scanning plane of the probe. Images are taken at up to 240 frames per second and so show the real-time motion of the heart. Motion (M-mode) produces a one-dimensional image showing how objects, such as the contracting ventricular walls, move towards or away from the plane of the probe over time. Pulse-wave Doppler mode illustrates the direction and magnitude of blood flow by releasing pulsed waves and measuring the time of the reflection to return to the transducer (Pichamuthu, 2009). Furthermore, an ECG scan was recorded in real-time in conjunction with the ultrasound images illustrating the electrical activity of the heart.

The heart of each rat was visualized in two different planes: the long-axis view and the short-axis view. Long axis view shows a sagittal cut through the left ventricle, a portion of the left atrium and the right ventricle, the aorta, and the apex of the heart. Short axis view shows an axial cut through the ventricles.
The real-time microvisualization (RMV) scanhead used to investigate the adult rats was the RMV-716 (VisualSonics Inc., Toronto, ON). This scanhead has a centre frequency of 17.5 MHz and a focal length of 17.5 mm.

**Ultrasound Data Collection**

To ensure consistency between measurements, each heart image was taken by aligning specific landmarks on the ultrasound screen. In long axis view, the probe was aligned so there was horizontal plane between the aorta and the apex of the heart across the screen. A B-mode scan was captured of the heart in this orientation for structural assessment including identification of a potential VSD. The orientation of the heart in this view prevented the use of M-mode for confirmation of the VSD as was done in fetal rats (Purssell *et al.*, 2012). An M-mode scan was taken with the plane of the probe across the contracting ventricular walls for assessment of contractility. For this M-Mode scan, the probe was always aligned with the papillary muscles for consistency. Pulmonary flow was assessed using Pulse-wave Doppler as a measure of right ventricular function. The probe was placed over the pulmonary artery and the Doppler angle was aligned parallel to the blood flow. The probe was then aligned in the short axis view orientation. A B-mode scan was captured of the heart in this orientation, using the mitral valve in the centre of the contracting ventricular walls as a landmark. An M-mode scan was taken with the plane of the probe across the contracting posterior and anterior ventricular walls. These scans were repeated using the probe in free hand (as opposed to supported by the arm of the ultrasound apparatus). The rat’s right paws were disconnected from the ECG and the
rat was rolled onto its left side to expose the right side of the chest. Probing the heart at this angle helped reduce shadow from the lungs and the ribs.

**Ultrasound Data Analysis**

The ultrasound data was analyzed using the VisualSonics™ software. The integrity of the septum was superficially assessed from the long axis B-mode scan. An M-Mode scan was chosen for analysis based on clarity. Although we wanted to compare the M-mode images taken in long axis and short axis view using the different methods to ensure consistency of the contractility and dimension measurements, often only one of these scans would be clear enough for analysis. There was no consistency in which view or method produced the best image between the rats. In light of this, we chose the best representative M-mode image taken from each rat, despite the view or method used. A wall trace of the contracting ventricular walls encompassed three cardiac cycles and was repeated for four sections of the M-Mode scan for each heart using both the standard wall trace tool and the left ventricular inner diameter (LVID) and outer diameter (LVOD) tool. The trace allows the software to measure the left ventricular volume and diameter of each heart. The software uses these measurements to calculate stroke volume (µL), ejection fraction (%), fractional shortening (%), and cardiac output (mL/min) of each fetal heart. Stroke volume is the volume of blood pumped out of the ventricle with each beat (difference between the volume in systole and diastole). Ejection fraction is the fraction of the end-diastolic volume in the ventricles that is ejected with each beat (percent difference between the left ventricular volume in systole and diastole as a fraction of the volume in diastole). Fractional shortening is the fraction of the diastolic dimensions of
the ventricles that is reduced in systole (percent difference between the left ventricular
diameter in systole and diastole as a fraction of the diameter in diastole). Cardiac output
is the volume of blood pumped out of the heart per minute (stroke volume multiplied by
the heart rate determined from the ECG). The values calculated from the four wall traces
were averaged.

M-Mode and B-Mode were used to measure the dimensions of the heart walls.
The width (distance between the endocardium and the epicardium) of the anterior
(LVAW) and posterior walls (LVPW) and the inner diameter (LVID) of the left ventricle
were measured in both systole and diastole at the location of the probe in M-Mode. The
values of the wall traces were averaged. The software calculated the volume of the left
ventricle in systole and diastole (LV Vol;s and LV Vol;d) using the LVID measurements.
The average wall thickness was calculated in B-mode by tracing the endocardial and
epicardial walls in systole and diastole in both short axis and long axis view.

The Pulse-wave Doppler scan of the pulmonary artery was used to measure flow
out of the right ventricle. Using the software embedded in the ultrasound system, peak
ejection velocity (mm/s) was determined using the pulmonary valve peak velocity tool.
The peak height of 10 pulses were measured and averaged. The peak height of the
opposing regurgitation peak for 10 pulses was also measured and averaged. A trace of the
pulmonary flow peaks was made using the velocity time integral (VTI) tool and used to
calculate the mean pulmonary velocity. Lastly, the peak velocity measurements were
used to calculate the peak pressure (mmHg) through the pulmonary valve.

As the cohort of rats included both males and females, there were substantial body
weight differences within both the control and the treatment groups. The females
weighed between 369g and 502g and the males weighed between 747g and 800g. A larger rat would be expected to have a larger heart and greater contractile strength. Low postnatal survival prevented us from having equal numbers of males and females for comparison. Instead, to normalize for the differences in rat sizes, each listed parameter was divided by body weight. The ratio values were compared between the DMO-treated and the control rats using t-tests, although data tables report actual dimensions.

The ECG scan was analyzed quantitatively for irregularities in the cardiac cycle. Using the time scale located on the ECG, the R-R, P-R, and Q-T interval were analyzed manually. The R-R interval represents the time for one cardiac cycle. The P-R interval represents the time for the electrical signal to travel between the sinoatrial and the atrioventricular node and is indicative of AV nodal function. A prolonged P-R interval indicates AV block. The Q-T interval represents the time for depolarization and repolarization of the ventricles. Prolongation of the Q-T interval can lead to dysrhythmia. The Q-T interval was reported as the corrected Q-T calculated by dividing the Q-T interval by the square root of the R-R interval, to account for differences in heart rate. Additionally, the ECGs were analyzed qualitatively for abnormalities in the pattern of the electrical cycle.

RESULTS

Stratification

Our initial plan was to stratify newborn pups into four groups depending on treatment, septum patency at parturition, and persistence or resolution of the VSD four weeks postnatally; however, this proved unattainable for two reasons. Firstly,
unbeknownst to us, the high-resolution ultrasound scanning heads available in the imaging facilities were not of the correct frequency and depth of focus for rat pups. Secondly, the postnatal mortality was much higher than expected due to the fact that although the viability of DMO-treated pups on GD 21, one day prior to parturition, is only slightly decreased (Weston et al., 2011), the long-term postnatal viability had not previously been assessed. Figure 3.2 shows control survival close to 100% in contrast to the initial viability of DMO-treated pups, which is about 45% within 12 hours of parturition, and only 18% by postnatal day (PND) 14. These surviving pups had reduced body weight compared to controls (data not shown). We attempted to reduce animal use in our experiments by employing litters from dams that had previously undergone isoflurane treatment for the conduct of high-resolution examination of pups during gestation. Isoflurane treatment had no significant effect on control pup viability, but was embryolethal in DMO-treated pups by PND 2. In light of the observed embryotoxic effects of isoflurane exposure, we were hesitant to risk exposing the surviving pups to this treatment postnatally. Thus, initial stratification was not performed as planned.

**Assessment of adult heart function under basal conditions**

Figure 3.3 depicts the daily average heart rate (Panel A) and MAP (Panel B), determined in 30 second intervals every 12 minutes (for a total of 120 time points per animal per day), for the control and the DMO-treated animals over a 32 day period. Two-way ANOVA revealed that there was no statistical difference in the average daily heart rate (p > 0.05) between the two groups or over the experimental period. Although there was no statistical difference in MAP (p > 0.05) between the control and DMO-treated
Figure 3.2. Two week postnatal survival (percent per litter). The effect of the *in utero* DMO treatment regimen and the isoflurane exposure on pup viability is depicted. Almost 100% of control pups survived, regardless of exposure to isoflurane. DMO treatment alone allowed a postnatal survival rate of 18.2% after two weeks. Combined exposure to DMO and isoflurane was fatal by two days postpartum. *N* values represent the number of litters assessed. Offspring for postnatal functional assessment came from 2 control litters and 3 DMO-treated litters and were not exposed to isoflurane.
Figure 3.3. Average heart rate (Panel A) and mean arterial pressure (MAP) (Panel B) ± standard deviation (SD) for the control (CTL) and DMO-treated groups for each of the 32 study days. There were no statistically significant differences in either heart rate or MAP over the study period or between the treatment groups (Two-way ANOVA; \( p > 0.05 \)). \( N = 8 \) CTL, 5 DMO rats.
group, the average MAP was consistently 3 – 9 mmHg greater in the DMO-treated group suggesting it may be biologically relevant.

Surprisingly, activity levels in the DMO-treated group were significantly greater than in controls (Figure 3.4; p = 0.014). Thus, we further stratified our heart rate and MAP by activity level (Figure 3.5); our rationale being that greater cardiac demand might reveal differences between the treatment groups. Qualitative examination of heart rates showed that at low activity levels (less than activity level 18) there was no difference in the average heart rate between the groups; however, at activity levels greater than 18, the DMO-treated group appeared to have a greater heart rate for a given level of activity compared to controls (Figure 3.5 Panel A). The DMO-treated group also appeared to have greater heart rate variability than the controls at high levels of activity. As is evident from Figure 3.5 Panel B, the MAP of the DMO-treated group was greater than that of the control group at every activity level, but this difference increased at activity levels greater than 18.

To further explore the apparent difference in heart rate variability at high levels of activity, the beat-to-beat intervals were examined using Poincaré plots for time points in which the rat had a high level of activity (greater than 18 counts). Figure 3.6 illustrates representative Poincaré plots for a control (Panel A) and a DMO-treated (Panel B) rat at activity levels of 20 and 21, respectively. Panel A shows a plot of the 30 seconds of data collected for CTL6 on July 6 at 06h00. The points on this plot are clustered tightly together, indicating tight control of heart rate. A similar data set collected for DMO6 on July 14 at 06h00 demonstrates far greater heart rate variability. Moreover, examination of the differences in X and Y coordinates shows this is not the result of a normal rise or fall
Figure 3.4. Average activity ± standard deviation (SD) for the control (CTL) and DMO-treated groups for each of the 32 study days. The activity of the DMO-treated group was significantly greater than that of the controls (Two-way ANOVA; p < 0.05). N = 8 CTL, 5 DMO rats.
Figure 3.5. Average heart rate (Panel A) and mean arterial pressure (MAP) (Panel B) stratified by activity level. At low levels of activity, the average heart rate of the two groups were comparable; however, at levels of activity surpassing approximately 18 counts, the average heart rate of the DMO-treated group were greater than the controls (CTL). The average MAP of the DMO-treated group was consistently greater than that of the controls. \( N = 8 \) CTL, 5 DMO rats.
Figure 3.6. Representative Poincaré plots for a control (Panel A) and a DMO-treated (Panel B) rat during a period of high activity (20 and 21 activity counts, respectively). Poincaré plots illustrate the heart rate variability. Each R-R interval (ms; RRI\textsubscript{n}) is plotted against the subsequent R-R interval (ms; RRI\textsubscript{n+1}) for a 30 second period. Tightly clustered points on the plot illustrate a controlled heart rate. Widely spread points indicate the heart rate is variable.
of heart rate (as physiological changes in heart rate yield a slope of 1). These plots suggest that cardiac function was impaired under conditions of increased cardiac load.

Hypervariability in heart rate (standard deviation greater than 5% of the mean heart rate over the 30 second period) was assessed as a crude indicator of dysrhythmia. Thus, the coefficients of variation were calculated for each time point (00h00, 06h00, 18h00) to quantitatively assess the heart rate variability in the control and treated rats. There was no difference in the average coefficient of variation between the control and treated groups (t-test; p > 0.554); however, a comparison of the incidence of episodes of hypervariability (Figure 3.7) revealed a significantly greater number of hypervariable periods in the DMO-treated group (Fischer’s Exact Test; p < 0.05). The differences in the mean group incidence of heart rate hypervariability were primarily attributable to two animals (DMO5 and DMO6), which displayed incidences of hypervariability of 43.6% and 44.7%, respectively. This suggests that for this parameter, these rats were the most severely affected by the DMO treatment in utero.

Stratification of the data by time point (00h00, 06h00, 18h00) revealed that the average heart rate was affected by the time of day (Two-way ANOVA; p < 0.0001). The heart rates were greatest at 6am and lowest at 6pm.

**Assessment of adult heart function under anaesthesia**

To further interrogate the effects of in utero exposure to DMO, animals were anaesthetized and cardiac function assessed using high-resolution ultrasound and ECG. There was no significant difference in stroke volume, ejection fraction, or fractional shortening between the control and DMO-treated groups after normalization for body
Figure 3.7. Assessment of the percent incidence of episodes of heart rate hypervariability for each of the DMO-treated and control (CTL) rats. Hypervariability: standard deviation greater than 5% of the mean heart rate. Normal variability: standard deviation less than 5% of the mean heart rate. The patterned bottom portion of the bar depicts the incidence of normal variability. The top solid portion of each bar depicts the incidence of hypervariability, the value of which is indicated for each rat. The combined incidence of hypervariability of DMO-treated rats was significantly greater than that of the control rats (Fischer’s Exact Test; $p < 0.05$). Each bar represents an assessment of 94 time points.
weight (Table 3.1). The mean cardiac output of the DMO-treated group was approximately twice that of the controls, but was not statistically different (Table 3.1). There was no significant difference in the peak or mean pulmonary velocity, the regurgitation velocity, or the peak pulmonary valve pressure between the control and DMO-treated rats (data not shown); however, this may reflect the inability to capture a clear Pulse-wave Doppler scan for three of the six DMO-treated rats.

ECG readings were obtained during the ultrasound procedure for each rat. The readings were examined to determine if there were any abnormalities in cardiac electrical activity. There was no significant difference in the mean R-R, P-R, or corrected Q-T intervals between the control and DMO-treated groups (Table 3.2). Despite the lack of quantitative differences between the groups as a whole, qualitative analysis of the ECGs revealed a cohort of rats in the study population were experiencing abnormal electrical activity (Figure 3.8). The ECGs from the control rats were normal with the exception of CTL1. A representative control ECG from CTL3 is shown in Panel A. The ECG recorded from CTL1 displayed an indistinct QRS complex and an abnormally large T wave (not shown). The ECGs from five of the six DMO-treated rats were abnormal; the ECG from DMO6 appeared normal (not shown). DMO1 (Panel B) had a very flat QRS complex, indicating less electrical activity through the ventricles. The accompanying M-mode images showed an abnormally sharp peak of contraction of the epicardial wall of the left ventricle (not shown). DMO2 (Panel C) had an extremely large T wave and a reduced QRS complex. DMO3 (Panel D) had a saw-tooth wave pattern in between QRS complexes, indicating abnormalities in the electrical activity through the atria. Furthermore, DMO3 experienced periodic episodes of what we interpreted to be a
B-mode was used to orient the heart in long axis view and then short axis view. An M-mode scan was taken across the contracting ventricular walls in both views and the clearest scan was selected for analysis of cardiac contractility. Software tools were used to trace the ventricular walls of the M-mode scan and the stroke volume, ejection fraction, fractional shortening, and cardiac output were calculated from the resulting trace data. T-tests were used to compare the mean values of the DMO-treated and control rats after normalizing for body weight. There were no statistically significant differences. The values presented in this table are the mean values before normalization to illustrate the true values for each cardiac parameter. \( N = 8 \) CTL, 6 DMO rats.

<table>
<thead>
<tr>
<th></th>
<th>Stroke Volume ((\mu)L)</th>
<th>Ejection Fraction (%)</th>
<th>Fractional Shortening (%)</th>
<th>Cardiac Output (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>304 ± 73.8</td>
<td>86.0 ± 6.5</td>
<td>58.4 ± 7.8</td>
<td>113 ± 35</td>
</tr>
<tr>
<td>DMO</td>
<td>342 ± 112.8</td>
<td>79.5 ± 8.0</td>
<td>51.4 ± 9.5</td>
<td>254 ± 107</td>
</tr>
</tbody>
</table>
The R-R, P-R, and Q-T intervals were measured manually on the ECG captured during ultrasound assessment for each rat. The R-R interval measures the total time of a cardiac cycle. The P-R interval reflects the time the electrical activity takes to travel from the sinoatrial node to the atrioventricular node. The Q-T interval reflects the time for depolarization and repolarization of the ventricles. A prolonged Q-T interval is a risk factor for ventricular tachycardia and sudden death. T-tests were used to compare the mean values of each interval for the DMO-treated and control rats. There were no statistically significant differences. \( N = 8 \) CTL, 6 DMO rats.

<table>
<thead>
<tr>
<th></th>
<th>R-R Interval (ms)</th>
<th>P-R Interval (ms)</th>
<th>Corrected Q-T Interval (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>178.6 ± 17.8</td>
<td>54.1 ± 3.8</td>
<td>3.1 ± 0.23</td>
</tr>
<tr>
<td>DMO</td>
<td>193.0 ± 31.2</td>
<td>54.9 ± 9.0</td>
<td>3.3 ± 0.72</td>
</tr>
</tbody>
</table>

Table 3.2. Quantitative assessment of electrocardiogram tracings
Figure 3.8. Representative electrocardiograms of adult rats exposed *in utero* to DMO or vehicle (CTL). A representative control electrocardiogram image is shown from CTL3 (Panel A). DMO1 (Panel B) had a flat QRS complex, indicative of reduced electrical activity through the ventricles. DMO2 (Panel C) also had a reduced QRS complex as well as an unusually large T wave. DMO3 (Panel D) experienced periodic episodes of premature ventricular contraction. DMO4 (Panel E) and DMO5 (Panel F) depict two different patterns of saw-tooth wave.
premature ventricular contraction. In these episodic pulses, the QRS complex was over twice as large as that of a regular pulse, not preceded by a P-wave, and not in rhythm with the other electrical cycles. The frequency of these episodic pulses increased significantly over a 10 minute observation period and each incidence coincided with a double peak on the accompanying M-mode image of the contracting ventricular walls, indicating the electrical abnormality had spread into the ventricles. Five minutes later (a total of 15 minutes), the M-mode showed that the rate of ventricular contractions had become extremely rapid. The ECG was no longer attached at this point, but the M-mode indicates that this heart was in ventricular tachycardia. DMO4 (Panel E) had an extreme saw-tooth wave appearance with an indistinct P and T wave and a very flat QRS complex. As the accompanying M-mode image showed normal ventricular contractions, this ECG most likely indicates that the electrical disturbances are within the atria, although the reduced QRS complex indicates there is less electrical activity in the ventricles as well. DMO5 (Panel F) had a large saw-tooth wave but with a distinct QRS complex and a distinguishable P wave; however, 15 minutes later the saw-tooth wave disappeared and the ECG appeared normal with a distinct P wave, T wave, and QRS complex, indicating autocorrection of the electrical activity in the atria.

**Assessment of adult heart structure**

Having assessed cardiac function we also examined the structure of the hearts using high-resolution ultrasound. The mean heart dimensions for each treatment group are shown in Table 3.3. After normalizing for body weight, the analysis revealed that there was no significant difference in the diameter of the left ventricular anterior wall.
B-mode was used to orient the heart in long axis view and short axis view. The Visualsonics™ software was used to trace the endocardial and epicardial walls in systole and diastole in both views to calculate the average wall thickness. An M-mode scan was taken across the contracting ventricular walls in both views and the clearest scan was selected. The software was used to measure the LVID, LVAW, and LVPW and to calculate the LV volume in both systole and diastole. T-tests were used to compare the mean values for the DMO-treated and control rats after normalizing for body weight. The values presented in this table are the mean values before normalization to illustrate the true dimensions of the hearts. Treatment effect: *significant difference between DMO-treated and control group (t-test; p < 0.05). LVID, left ventricular inner diameter; LVAW, left ventricular anterior wall; LVPW, left ventricular posterior wall; LV Volume, left ventricular volume. N = 8 CTL, 6 DMO rats.

<table>
<thead>
<tr>
<th></th>
<th>LVID (mm)</th>
<th>LVAW (mm)</th>
<th>LVPW (mm)</th>
<th>LV Volume (μL)</th>
<th>Wall Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic</td>
<td>Diastolic</td>
<td>Systolic</td>
<td>Diastolic</td>
<td>Systolic *</td>
</tr>
<tr>
<td>CTL</td>
<td>3.3 ± 1.0</td>
<td>7.9 ± 1.2</td>
<td>3.6 ± 0.69</td>
<td>1.9 ± 0.56</td>
<td>4.1 ± 0.89</td>
</tr>
<tr>
<td>N=8</td>
<td></td>
<td></td>
<td>Systolic</td>
<td>Diastolic</td>
<td>49.2 ± 35.2</td>
</tr>
<tr>
<td>DMO</td>
<td>4.5 ± 1.6</td>
<td>9.0 ± 1.6</td>
<td>3.8 ± 0.31</td>
<td>1.9 ± 0.27</td>
<td>3.7 ± 0.58</td>
</tr>
<tr>
<td>N=6</td>
<td></td>
<td></td>
<td>Systolic</td>
<td>Diastolic</td>
<td>106 ± 73.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diastolic</td>
<td>2.36 ± 0.25</td>
</tr>
</tbody>
</table>

Table 3.3. Assessment of heart dimensions
(LVAW) or the left ventricular posterior wall (LVPW) in systole or diastole indicating that there was no difference in wall thickness at the location of the probe. Similarly, there was no significant difference in the calculated average wall thickness. There was a trend towards an elevation in the LVID in the DMO-treated rats, although this did not quite reach significance ($p = 0.063$). The volume of the left ventricle in systole was significantly larger than that of the control ($p = 0.023$), although there was no statistical difference in diastolic volume.

The preliminary investigation of the integrity of the interventricular septum in B-mode revealed no obvious persisting VSDs with the exception of DMO5 in which there was a hole in the muscular region of the septum.

**DISCUSSION**

Experiments in mutant mouse models (Pashmforoush et al., 2004; Zhu et al., 2008) and clinical findings in patients with identifiable mutations of critical cardiac genes (Holt and Oram, 1960; Schott et al., 1998; Benson et al., 1999) demonstrate a clear relationship between structural defects such as VSD and functional pathologies postnatally. This lead us to hypothesize that an *in utero* chemical exposure causing VSD should induce functional deficits that persist into adulthood. Indeed, the novel and key finding of the current study is that *in utero* exposure to DMO using a treatment regimen that induces a 74% incidence of VSD (Weston et al., 2011) is also associated with significant pathophysiological sequelae that present one year postnatally, most notably abnormalities in cardiac conduction resulting in rhythm disturbances.
Perhaps the most disturbing observation of this study is that all but one of the DMO-exposed rats had no obvious structural anomalies at the termination of the study, yet presented with profoundly abnormal ECG patterns. This finding is novel because functional deficiencies were previously considered to be secondary to a congenital heart defect (Mitchell et al., 1971). Although technical limitations prevented us from determining whether these animals had a VSD at birth that resolved postnatally or whether individual animals were born with an intact ventricular septum, this observation has three potential implications.

Firstly, if an animal was born with a VSD that resolved postnatally, it demonstrates in experimental animals that even a resolved VSD is associated with a significant health risk. This is probably translatable to humans whose VSD has resolved within the first year of life. Unlike patients with repaired VSD who are monitored closely by cardiologists specializing in CHD, most patients with VSDs that have spontaneously resolved are lost to clinical follow-up and are unaware of their potential insidious health risk because the prevailing thought is these patients are no longer at risk.

The second implication relates to the possibility that the rats treated with DMO in utero were born with an intact septum. This indicates functional anomalies may occur in the absence of structural defects, which counters the prevailing dogma (Mitchell et al., 1971). Moreover, because we have identified rat fetuses on GD 21, one day prior to parturition, with functional deficits of the heart but an intact ventricular septum, it suggests some of the animals in the current study may have been born with functional disturbances, rather than having developed them postnatally.
The third implication of cardiac functional deficits in the face of apparently normal heart morphology relates to the conduct of preclinical regulatory safety studies that only assess the ability of a drug or chemical to induce structural defects. We showed clear functional pathologies in the absence of structural defects indicating that preclinical regulatory developmental toxicity endpoints may need to include functional measures to be sufficiently sensitive to identify the teratogenic potential of a chemical.

An important observation that needs further investigation is the variability in the severity of the pathophysiological outcomes after DMO exposure. We speculate the reason for the differential susceptibility may be rooted in variable effects of DMO exposure on the expression profile of genes critical to heart development. By way of illustration, using whole mount in situ hybridization, we have observed DMO-induced ectopic expression of NPPA in the right ventricle on GD 12, 24 hours following the last dose of DMO; however, the extent of invasion into the right ventricle and the intensity of staining is varied between embryos (Ozolinš, unpublished results). Thus, since gene expression can be variably affected by in utero DMO exposure, we anticipate the pathophysiological outcomes are similarly variable between DMO-treated animals. This is supported by the variety of conduction disturbances that were observed in the in utero DMO-treated adult rats. For example, the ECGs of three of the DMO-treated rats displayed a saw-tooth wave indicative of excessive atrial activity (atrial flutter or fibrillation). These disturbances have been associated with misexpression of connexin proteins including cx40 (Bevilacqua et al., 2000; Ryu et al., 2007; Xing et al., 2011), a downstream target of both Tbx5 and Nkx2.5 (Bruneau et al., 2001; Takeuchi et al., 2003). We speculate that the levels of cx40 expression in these three animals will differ
not only from the controls, but also from the other DMO-treated rats that did not display this ECG wave. Additionally, the ECG of one adult rat captured an incident of premature ventricular contraction leading to ventricular tachycardia, a pathology that has been linked to minK function (Vohra, 2007), a target of Nkx2.5 (Jay et al., 2004; Pashmforoush et al., 2004) whose expression is altered by DMO (Ozoliņš, unpublished results). Again, we speculate that in utero DMO exposure affected the expression of minK, thereby predisposing the animal to abnormalities in myocardial repolarization leading to dysregulation of electrical conduction.

The electrical disturbances found in the ECGs of the DMO-treated animals are corroborated by findings from the telemetry analysis. The DMO-treated rats had an increased incidence of episodes of heart rate variability, although this may be partially attributed to their observed greater physical activity. Stratification by activity level and Poincaré plots revealed that the DMO-treated rats had a greater and more variable heart rate at high levels of activity, suggesting that these rats are at an increased risk for experiencing dysrhythmia when the heart is stressed. In addition, although the anticipated decrease in cardiac output is not apparent at basal conditions, it may become evident when the animal is subjected to heightened cardiac load. Thus, our experimental results appear to be consistent with clinical data suggesting there is a higher incidence of conduction disease and exercise intolerance found in patients born with a CHD (Blake et al., 1982; Kidd et al., 1993; Fukuda et al., 2002; Roos-Hesselink et al., 2004; Walsh and Cecchin, 2007; Liberman et al., 2008; Roos-Hesselink and Karamermer, 2008).

Although there was no statistically significant difference in MAP between the DMO-treated and control rats, the average MAP of the DMO-treated group was
consistently greater than that of the control group by 3 – 9 mmHg. This difference was maintained even following stratification for activity level, suggesting the rise in MAP is of biological significance. Further corroboration of the biological relevance of the increased MAP comes from a meta-analysis study of human populations, demonstrating an increase of 3 – 4 mmHg systolic blood pressure translates population wise into a 20% higher stroke mortality and a 12% higher mortality from ischemic heart disease (Lewington et al., 2002). It has been shown previously that the DMO treatment used in this study induced the ectopic expression of NPPA in the right ventricle of the GD 12 rat heart (Ozolinš, unpublished data). NPPA plays a role in blood pressure regulation (Needleman et al., 1985). Misexpression of this gene during development may have lasting effects on blood pressure control. In heterozygous Tbx5 knockout mice, NPPA was ectopically expressed during development but the levels normalized in adulthood (Bruneau et al., 2001). Studies are underway to determine whether NPPA levels normalize postnally in adult rats exposed to DMO \textit{in utero}, and if this is related to the increased MAP observed in the DMO-treated rats.

Surprisingly, the DMO-treated rats were significantly more active than the control rats. There have been previous reports of various teratogenic exposures inducing hyperactivity in offspring. For example, maternal ethanol consumption in a Dunkin-Hartley guinea pig model increased spontaneous locomotor activity in offspring (Shea et al., 2012), an effect that is also found in humans with fetal alcohol syndrome (Driscoll et al., 1990). Developmental delay and CNS dysfunction has been described as a symptom of TMD syndrome (Zackai et al., 1975; Feldman et al., 1977). These results are also consistent with studies in rats demonstrating that 250mg/kg/day TMD administered from
GD 7 – GD 11 causes subtle but distinct neurobehavioral consequences that included decreased swimming ontogeny and spontaneous alteration behavior (Vorhees 1983, 1985).

Ultrasound was used to assess heart dimensions, contractility, and pulmonary flow to further characterize heart function. There did not appear to be differences in the wall thickness as found by ultrasound but these morphometric measurements are being confirmed histologically. The left ventricular volume was greater in the DMO-treated hearts while in systole, possibly indicating a reduction in the ability of the ventricle to fully contract. Unexpectedly, the cardiac output appeared to be greater in the DMO-treated hearts. Additionally, although we expected a rise in pulmonary pressure characteristic of Eisenmenger’s syndrome, there was no difference in the any measurements of pulmonary flow.

Only one fifth of the DMO-treated pups (that were not exposed to isoflurane) survived into adulthood. The pups had a lower body weight and were not as robust as the control pups. The low postpartum survival was an unexpected finding for two reasons. First, previous studies from our laboratory only showed minor differences in viability between control and DMO-exposed pups at parturition, although two hour postpartum survival was not assessed (Weston et al., 2011). Second, exposure to TMD, the parent compound of DMO, had approximately 75% survival at weaning (PND 21) despite 49% incidence of VSD at parturition (Fleeman et al., 2004). Ad hoc necropsies of dead DMO-exposed pups did not show a relationship with VSD or outflow tract anomalies, but rather with an unusual lung phenotype. Formal ongoing studies suggest defects in lung maturation may account for the poor viability of DMO-exposed pups. This unexpectedly
low postnatal survival has significant implications for our future studies and will require us to modify our DMO treatment paradigm to ensure higher postnatal survival. Furthermore, none of the pups that were exposed to both DMO and isoflurane survived past PND 2, suggesting that high-resolution ultrasound may not be as non-invasive as we previously reported (Purssell et al., 2012). Future studies will require the use of an alternate anaesthetic in disease models.

The surviving pups exposed to DMO were less robust than controls and therefore we did not use ultrasound at parturition to determine the patency of the ventricular septum. Although the inability to assess the status of the septum at parturition makes for a less powerful study, our observations nevertheless provide novel and important findings that may be of clinical significance. Foremost of our findings is that a brief chemical exposure in utero may produce functional deficits of the heart that are present in the adult rat. Moreover, these functional deficits are evidence that in the absence of gross structural anomalies, it appears as though specific cohorts of animals are sensitive to these chemical exposures while others are resistant. Studies are ongoing in our laboratory to identify the reason for this variable sensitivity as we feel this may be a potential target for intervention strategies aimed at reducing the severity and frequency of environmentally mediated heart pathologies. This may become more important clinically because increased chemical exposure in the environment and increased use of drug therapy in pregnancy is predicted to cause an increase in the prevalence of CHD (van der Linde et al., 2011).
ACKNOWLEDGEMENTS

This study was conducted at Queen’s University with the financial support of a Queen’s University Senate Advisory Research Committee (SARC) Grant, and a grant from the Garfield-Kelly Cardiovascular Research Fund. We appreciate the excellent technical support provided by Kim Laverty of Dr. Michael Adams’ laboratory and the help of Amy Hilliard with the telemetry data analysis.
CHAPTER 4

DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS
Discussion

The goal of this work was to determine if an *in utero* chemical exposure capable of producing a VSD also induces functional pathologies, both during gestation and postnatally. The rationale for this investigation was that although VSDs account for almost one third of the incidence of birth defects (Dolk *et al.*, 2011), the full extent of the functional consequences of VSD has not been well explored in animal models. Clinically, it is clear that large non-resolving VSDs are associated with the development of cardiovascular disease including pulmonary arterial hypertension, Eisenmenger’s syndrome, and dysrhythmia, both in patients in which the VSD remains open and in those that undergo surgical repair (Roos-Hesselink *et al.*, 2004; van der Velde *et al.*, 2005; Duffels *et al.*, 2007; Engelfriet *et al.*, 2007; Roos-Hesselink and Karamermer, 2008). These pathologies can lead to functional limitations and even sudden cardiac death (Blake *et al.*, 1982; Roos-Hesselink *et al.*, 2004; Duffels *et al.*, 2007). While the postnatal pathologies of large persisting VSDs have been well described, they only account for 10 - 15% of VSD incidence. The remainder resolve spontaneously within the first year of life (Hoffman and Kaplan, 2002). There is a dearth of knowledge surrounding the functional implications in the majority of VSD cases due in large part to the loss of clinical follow-up upon spontaneous resolution (Meijboom *et al.*, 1994; Pierpont *et al.*, 2007).

My first hypothesis was maternal treatment of rats with clinically relevant exposures to DMO would cause persistent deficits in fetal rat cardiac function. To test this hypothesis, we exposed rats to dimethadione (DMO) during gestation, a chemical known to induce VSD. Assessment of cardiac function in fetal rats using high-resolution
ultrasound revealed that DMO-exposed fetuses had irregularities in heart rhythm, including reduced heart rate and an increased incidence of dysrhythmia. Additionally, histological analysis revealed cardiomyopathies such as spongiform myocardium, which coincided with reduced cardiac output and ejection fraction.

My second hypothesis was adult rats exposed to DMO during gestation would exhibit persistent deficits in postnatal cardiac function. Using the same dosing paradigm as was used to test the first hypothesis, we exposed rat embryos to DMO in utero and allowed them to litter out naturally. A cohort of pups were allowed to mature into adults and cardiac function was assessed using high-resolution ultrasound, telemetry, and electrocardiography. DMO-exposed adult rats experienced dysrhythmia due to disturbances in the electrical conduction in the heart. Additionally, these rats had a high mean arterial pressure and a greater activity level. Unlike in the fetal hearts, we did not observe a reduction in cardiac output postnatally as a result of exposure to DMO. We speculate this may be because pups with such hearts were non-viable. Overall, these results provide support for both hypotheses.

Although four of the five fetal rats that suffered dysrhythmia also had a VSD, one fetus had a complete septum, indicating that cardiac function may be disrupted in utero in the absence of a visible structural anomaly. Due to this observation and the fact that about 80% of TMD-induced VSD close spontaneously by weaning (Fleeman et al., 2004), it was my intention to stratify the DMO-exposed pups into the following groups: no VSD, VSD resolved, and unresolved VSD. Technical issues prevented this approach, and instead the patency of the ventricular septum was assessed in the adult animals using high-resolution ultrasound (to be confirmed histologically). The later assessment of the
integrity of the septum in the adult rats revealed that five of the six DMO-treated rats did not have a persisting VSD. Of these six rats, only one (without VSD) had normal ECG patterns, whereas the remaining four rats without VSD as well as the single rat with a persisting VSD displayed abnormalities in electrical conduction. These results are both novel and disturbing because they indicate that although functional pathologies often occur in conjunction with VSD, as has been recognized clinically, they can also exist in the absence of a severe structural defect. We believe these observations are not DMO-specific, but rather broadly applicable to other teratogens known to induce CHD. If these observations in rat are applicable to humans, there are several profound implications.

The first possible implication relates to patients who may have been exposed to potential teratogens in utero. My results suggest an infant could be born without readily detectible CHD, or with VSD that resolves within the first year of life, and still be at extreme risk for heart pathologies later in life. This indicates that our previous suggestion that VSDs may be a sentinel for serious functional anomalies may not necessarily be the case. Thus, there may be a cohort of individuals at risk for the development of severe cardiovascular disease that has previously been unrecognized. It is possible that idiopathic (not linked to specific mutations) sudden deaths in adolescents, especially in young athletes, may be a consequence of unrecognized heart pathologies. Furthermore, the increase of at-risk individuals in the population has implications for health care. The burden of cardiovascular disease on the health care system, especially as survivors of CHD age, could be larger than previously expected.

The second implication relates to the conduct of regulatory developmental toxicology studies to test the safety of industrial or pharmaceutical products. Currently,
assessment of the teratogenic effects of a chemical only considers its ability to induce structural defects of the heart. We have shown that DMO can induce functional pathologies in the absence of a structural defect, not only in utero but later in life, indicating that the current endpoints for chemical safety assessment may be insufficient. The endpoints of regulatory studies should be expanded to include assessment of cardiac conduction and contraction.

Additionally, the results of this study highlight the importance of identifying the biological targets of gestational exposure to DMO and other potential agents that induce CHD. The functional pathologies in the fetal and adult rats that were induced by in utero DMO exposure in this study mirror those that result from mutations in transcription factors that direct heart development and regulate genes critical to heart function. Furthermore, the expression of a number of genes is perturbed by DMO treatment and mutations of these same genes clinically or experimentally in mice result in structural and functional findings similar to what is noted after exposure to DMO. For example, the conduction defects found in both the fetal and adult rats could be explained by a disturbance in the expression of cx40 or minK. minK function is critical to myocyte repolarization (Li et al., 1996) and has been linked to Q-T interval prolongation (Splawski et al., 1997), a risk factor for cardiac arrhythmias including ventricular tachycardia (Vohra, 2007) which was observed in one of the in utero DMO-treated adult rats. Nkx2.5 misexpression has been shown to cause AV block both clinically and in a mutant mouse model (Benson et al., 1999; Jay et al., 2004; Pashmforoush et al., 2004). Similarly, Tbx5 misexpression results in downregulation of cx40 associated with AV block (Bruneau et al., 2001). Although there was no statistically significant difference in
P-R interval, the replacement of the P wave with a saw-tooth wave on the ECG of three of the in utero DMO-treated adult rats indicates there may have been impairment of the conductivity through the AV node. The spongiform myocardial phenotype observed in DMO-exposed fetal rat hearts (Weston et al., 2011; Purssell et al., 2012) may be linked to the DMO-mediated repression of MLC2v (Ozolinš, unpublished data), an important component of the myocardial wall. The reduction in cardiac output and ejection fraction found in the fetal rat hearts may be due in part to the malformed myocardial walls. Alternately, a disturbance in the expression of SERCA2a (positively regulated by Tbx5) which causes diastolic dysfunction in mice (Zhu et al. 2008) would contribute to this phenotype. Finally, the rise in blood pressure observed in the in utero DMO-treated adult rats may be indicative of a disruption in the expression of NPPA, a peptide hormone involved in blood pressure regulation (Needleman et al., 1985), which is downregulated in DMO-exposed embryonic hearts (Ozolinš, unpublished data). Understanding the targets and mechanisms of action of heart teratogens may provide the opportunity to develop intervention strategies to reduce the incidence and severity of CHD and related functional pathologies.

**Conclusion**

Large VSDs and those requiring surgical intervention require a lifetime of follow-up from congenital heart specialists; however, spontaneously resolving VSDs have previously been considered fairly innocuous as evident from the lack of clinical follow-up to such patients. The results of this research refute that dogma and demonstrate clearly in an animal model that gestational exposure to a CHD-inducing chemical can cause
profound functional abnormalities of the cardiac conduction system and the myocardium, even in the absence of a structural defect. Moreover, the results of this study indicate the importance of long-term follow-up for patients born with a VSD, even if it resolves spontaneously, and for those that are known to have been exposed to chemicals that induce CHD.

**Future Directions**

For the continuation of this work, a number of unanswered questions need to be addressed. First and foremost, the molecular mechanisms underlying our laboratory’s hypothesis regarding the association between treatments causing VSD and the functional defects should be investigated. There are many similarities between the structural and functional cardiac pathologies found in rats exposed to DMO in utero and the humans and mice carrying loss-of-function mutations of Tbx5, Nkx2.5, and GATA4. This suggests that the expression of these transcription factors, as well as their downstream targets which include cx40, NPPA, MLC2v, SERCA2a, and minK, may be disturbed as a result of DMO exposure. Preliminary qRT-PCR data from this laboratory supports that the expression of some of the aforementioned genes is altered in the DMO-treated fetuses (Ozolinš, unpublished data). These data should be expanded on by (1) examining a larger cohort of both fetal and adult rat hearts, and (2) determining whether the expression domains of these genes within the heart have been perturbed by DMO treatment, as ectopic expression patterns of many of the aforementioned genes are linked to CHD. In addition, to ensure a more robust study design, the samples should be stratified based on the presence or absence of both structural anomalies and functional deficits.
Furthermore, this laboratory is interested in the mechanism by which DMO induces changes in the expression of genes critical for heart development. More specifically, it would be interesting to test the hypothesis that DMO mediates its deleterious effects on gene expression by altering the epigenome. The epigenome controls the three-dimensional structure of DNA through a number of mechanisms including specific chromatin remodeling proteins as well as the acetylation and methylation of specific regions of both histones and DNA (reviewed in Bernstein et al., 2007; Szyf, 2009). Alterations to the epigenome can modulate gene expression by altering the accessibility of transcriptional machinery to the DNA. Epigenetic factors play an important role in heart development (Weston et al., 2006; Bruneau, 2010; Takeuchi et al., 2011). This laboratory has already found evidence that DMO has HDAC inhibitory properties (Ozolinš, unpublished data). HDAC inhibition would result in an elevation in histone acetylation, which generally is associated with chromatin unwinding and increased gene transcription. This might explain the results from whole mount in situ hybridization experiments which show increased expression of NPPA in the right ventricles of DMO-exposed rats 24 hours after the last dose of DMO (Ozolinš, unpublished data).

Due to the long-term persistence of the functional deficits that were observed in this study, it would be interesting to investigate if DMO has the ability to interfere with DNA methylation, the most enduring form of epigenetic regulation (reviewed in Bernstein et al., 2007). Increased DNA methylation typically causes the chromatin to wrap up more tightly and therefore prevents transcriptional machinery from binding the DNA. The critical target of DNA methylation is often the gene promoter, as this region
controls the initiation of gene transcription. I have optimized a bisulfite sequencing assay for the investigation of DNA methylation in the promoters of Tbx5 and Nkx2.5. If the epigenome is the primary target of DMO, the pattern of epigenetic changes would support the differences in gene expression caused by in utero DMO treatment.

More importantly from a clinical perspective, if the epigenome is disrupted by DMO or other heart teratogens, it may be a useful target for intervention strategies to reduce the risk of heart pathologies in at-risk individuals. This is possible because unlike the genetic code, the epigenome may be sculpted by environmental factors including diet and lifestyle (reviewed in Szyf, 2009). For example, epidemiological studies reveal that mothers consuming diets low in methyl donors or who have loss-of-function mutations in genes important in regulating the methyl donor pool are at increased risk for giving birth to infants with CHD and likely the associated pathophysiological consequences (Christensen et al., 2009). Contrastingly, diets rich in methyl donors have a profound effect on DNA methylation and more importantly, a resultant visible phenotype in mice (reviewed in Bernal and Jirtle, 2010). If in utero DMO exposure does in fact influence DNA methylation, this suggests it would be useful to test the hypothesis that a diet rich in methyl donors protects against teratogen-induced disruption of DNA methylation patterns on the promoters of genes critical to heart development and in doing so may also protect against the pathophysiological consequences.

In addition, the adult rat heart ultrastructure should be investigated using histology. Although analysis of the wall thickness of the adult rat hearts using high-resolution ultrasound did not reveal any differences between the DMO-treated and the control rats, these findings need to be confirmed using histological sections as the
ultrasound images in the adult rats were confounded by shadows and reflections that were more disruptive in the larger animals. This may have prevented accurate analysis of the heart dimensions. Furthermore, histological analysis will allow the assessment of the compaction and trabeculation of the myocardium, which are both critical to the ability of the heart to generate appropriate contractile force. MLC2v, which is regulated by Tbx5 and Nkx2.5 (Lyons et al., 1995; Ghosh et al., 2001), plays a role in the formation of the trabeculae in the myocardial walls. If DMO is interfering significantly with the MLC2v expression, abnormalities of the trabeculae will be visible histologically. Furthermore, DMO treatment induces a spongiform myocardial phenotype in fetal rat hearts (Weston et al., 2011; Pursell et al., 2012), a condition that can be caused by decreased levels or loss-of-function of the myosin heavy chain gene 7 (Budde et al., 2007). Using whole-mount in situ hybridization, this laboratory has demonstrated significant reductions in myosin heavy chain 6 expression in rat embryonic heart 24 hours after the last dose of DMO (Ozolinš, unpublished data). It would be useful to know whether other adult forms of cardiac-specific myosins are also downregulated after in utero exposure to DMO.

Lastly, this laboratory is interested in expanding on the telemetry data that was collected for this work, which was in unstressed, undisturbed rats. New rat telemeters that have ECG capability are being purchased that will allow monitoring of the electrical activity in the rat hearts without anaesthesia over a continuous period to further characterize the frequency and severity of electrical disturbances. Additionally, telemetry can be used to investigate the ability of the cardiovascular system to function under various conditions. In view of our current observations showing that the heart rate of the DMO-treated rats was greater and more irregular than controls at high levels of activity,
it would be interesting to assess the cardiac function of the DMO-treated rats at controlled levels of activity using rat treadmills. Toward this end, I have already begun to assess the effect of sympathetic drive on cardiovascular parameters using the acoustic startle test. This test involves exposing the DMO-treated rats to a short 90dB noise to activate the sympathetic nervous system and assessing the cardiovascular response.

Finally, to further investigate the blood pressure differences that were seen in the DMO-treated rats, I have conducted a high and low salt challenge to determine if the DMO-treated rats have differences in salt sensitivity, which might suggest predisposition to hypertension or perhaps chronic kidney disease. The data from these experiments remains to be analyzed.

Together these proposed studies would shed more light into the long-term effects of \textit{in utero} chemical exposure and would provide potential targets for intervention strategies designed to reduce the incidence and/or severity of structural defects such as VSD and pathophysiological disturbances of heart function.
REFERENCES


