PERFLUOROOCTANOIC ACID-INDUCED DEVELOPMENTAL CARDIOTOXICITY IN AN AVIAN MODEL

by

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Perfluorooctanoic acid (PFOA) is a synthetic chemical used in polyfluormer production. It is a widespread environmental contaminant and is known to induce developmental toxicity in rodent models. As several related compounds also induce developmental cardiotoxicity in avian models, a series of experiments were carried out in chicken embryos and hatchling chickens to assess the effects of PFOA on developing hearts and to explore underlying mechanisms. Histology was used to assess late stage embryo hearts and echocardiography was used to assess morphological and functional parameters of hearts in living one-day-old hatchlings. Hearts of exposed embryos had a thinned right ventricular wall and multiple morphological and functional changes were detected in the left ventricles of exposed hatchlings. As PFOA is known to activate the peroxisome proliferator activated receptor α (PPARα) in rodent models, the PPARα agonist WY 14,643 was used to assess the role of PPARα in PFOA-induced developmental cardiotoxicity. Real-time polymerase chain reaction (PCR) and western blot were used to assess hearts of early stage embryos for expression of genes related to heart development. WY 14,643 partially mimicked the morphological and functional effects of PFOA only in the left ventricle of hearts of hatchling chickens and bone morphogenic protein 2 (BMP2) signaling pathways were involved in mediating the process. Additionally, primary cardiomyocyte cultures were used to

explore mechanisms of toxicity at the cellular level. Viability, contractility, and reactive oxygen species (ROS) generation were evaluated in primary cardiomyocytes isolated from hearts of embryos exposed to PFOA in ovo or were exposed in vitro. Primary cardiomyocytes isolated from embryos exposed to PFOA in ovo functioned similarly to hearts of exposed hatchling chickens. Primary cardiomyocytes exposed in vitro responded differently, suggesting that the cardiotoxicity is likely from interference of PFOA in an early stage of heart development rather than from overt cytotoxicity to cardiomyocytes. In conclusion, this dissertation work indicates that PFOA induces morphological and functional alterations in hearts of chicken embryos and hatchling chickens, that mechanisms of the cardiotoxicity may involve PPARα and BMP2 signaling pathways, and that PFOA is likely interfering in an early stage of heart development.

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A Dissertation

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by

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α	alpha	5
β	beta	6
μm	micrometer	21
ANOVA	one-way analysis of variance	25
BMP2	bone morphogenic protein 2	6
BMPR1A	bone morphogenic protein receptor type IA	54
CHD	congenital heart disease	9
DCF	2',7'-dichlorofluorescein	81
DMSO	dimethyl sulfoxide	81
GATA4	transcription factor GATA-4	6
HBSS	Hank's Balanced Salt Solution	79
IL-1β	interleukin 1 β	13
IL-6	interleukin 6	13
L-FABP	liver fatty-acid-binding protein	53
LITAF	LPS induced TNF α like factor	54
mg	milligram	20
ml	milliliter	23
mM	millimolar	23
mm	millimeter	20
Nkx2.5	homeobox protein Nkx-2.5	6
PFAAs	perfluoroalkyl acids	1
PFOA	perfluorooctanoic acid	1

PPARα	peroxisome proliferator activated receptor α	5
PTFE	polytetrafluoroethylene	1
RIPA buffer	radio-immunoprecipitation assay buffer	54
ROS	reactive oxygen species	12
RPM	revolutions per minute	24
RXRα	retinoid X receptor α	54

CHAPTER 1 INTRODUCTION

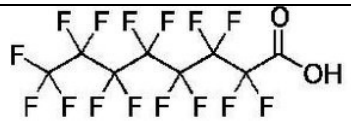
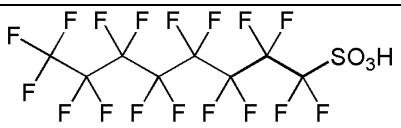
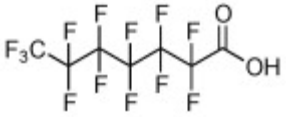
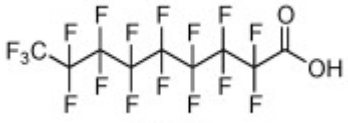
1.1 Basic information on PFOA

Perfluorooctanoic acid (PFOA) is a fluorinated chemical that belongs to the perfluoroalkyl acid (PFAAs) family of compounds. Chemically, PFOA is composed of an eight-carbon atom chain, with one carbonyl group attached to one end and fluorine connected to all the carbons (Table 1.1). The carbon-fluorine bond is among one of the strongest chemical bonds, making PFOA highly stable, both in the environment and biota. PFOA, along with other perfluorinated compounds such as the fluorotelomers, was used as to make water and oil repellent coatings for textiles such as fabrics and leather (Fiedlera et al. 2010). The current major use of PFOA is as a synthesis aid in the production of fluoropolymers, such as polytetrafluoroethylene (PTFE), polyvinylidene fluoride, and fluoroelastomers. The fluoropolymers are then used in myriad industrial and consumer products such as automobiles, computers, non-stick cookware, and many others.

1.2 Rationale of investigating the toxicity of PFOA

The plastics industry began using PFOA in early 1950s (Emmett et al. 2006); since then, its use has expanded and it has been released into the environment through legal discharge permits, accidentally through spills, and as a breakdown product of other fluorinated compounds (Zhu and Lin. 2008; Frömel and Knepper. 2010). Due to its high stability in environmental media and biota, it is ubiquitously detectable around the world. Because of its extreme stability

Table 1.1 Chemical structure of PFOA and related perfluorinated compounds.

Chemical name	**Structure**
Perfluorooctanoic acid (PFOA)	
Perfluorooctanesulfonic acid (PFOS)	
Perfluorohexanoic acid (PFHxA)	
Perfluoroheptanoic acid (PFHpA)	
Perfluorononanoic acid (PFNA)	
8:2 fluorotelomer alcohol (8:2 FTOH)	

and resistance to metabolic, photochemical, and microbial breakdown, it was previously considered nearly toxicologically inert; however, being chemically inert does not mean that it is unable to interfere with biological systems. Increasing concerns about its negative effects on human, wildlife, and ecosystem health arose between the 1980s and 1990s, and eventually lead to a stewardship program between the United States Environmental Protection Agency (USEPA) and eight major US manufacturers of PFOA to eliminate PFOA and related compounds from emissions and products by 2015 (USEPA 2013). As monitored by the Centers for Disease Control and Prevention (CDC), concentrations of PFOA in the serum of the general human population are decreasing. In samples collected between 2006 and 2007, the median concentration in the general human population was 4.3 ng/ml (CDC 2011); in samples collected between 2009 and 2010, the median concentration was 3.2 ng/ml (CDC 2013). However, exposure to PFOA likely will continue for a considerable amount of time as the half-life of PFOA in humans is up to several years. PFOA also is a breakdown product of certain fluorinated telomer alcohols and other precursor compounds of fluorinated polymers (Wang et al. 2005) and will likely continue to contaminate the environment. Additionally, the stewardship program to eliminate PFOA from emissions and products is limited to the US; production could still occur in other countries. As a result, it is necessary to investigate potential toxicities of PFOA, which will provide data to regulatory agencies for establishing safe exposure standards and/or minimizing the risk to exposed populations.

1.3 Levels of PFOA exposure in humans

PFOA exposure to the general human population, as previously mentioned, is at a relatively low level. Current data from the CDC indicates that the median serum concentration of

PFOA in the general human population was 3.2 ng/ml in samples collected between 2009 and 2010 (CDC 2013). The full exposure pathway in the general human population has not been fully characterized. However, contaminated drinking water in areas surrounding fluorochemical plants and occupational exposures lead to much higher exposure levels in certain populations relative to the general human population. In an area of West Virginia contaminated with PFOA by a manufacturing plant, epidemiological studies of the exposed populations reported a median PFOA serum concentration of 26.6 ng/ml, with a high value of 17,556.6 ng/ml (Steenland et al. 2009). Another study reported a median serum value of 75.7 ng/ml in residents of communities surrounding a fluoropolymer production facility (Hoffman et al. 2011). Serum concentrations as high as 5100 ng/ml have been reported in occupationally-exposed humans (Olsen et al. 2007). PFOA exposure potentially poses a much higher health risk to these populations and additional data on the toxicity of PFOA will help to protect these susceptible populations.

1.4 Adverse health effects associated with PFOA exposure

Epidemiological studies of the effects of PFOA on human development have been carried out in areas of known high exposures such as West Virginia-Ohio (US) and the Faroe Islands (Denmark). Fluorochemical plants contaminated the drinking water in the West Virginia-Ohio area. The Faroe Islands population was exposed to PFOA due to their frequent consumption of marine mammals (Weihe et al. 2008). In the West Virginia-Ohio population, modest associations of PFOA serum concentrations with preeclampsia and birth defects were found (Stein et al. 2009). PFOA exposure was associated with elevated serum cholesterol and uric acid levels in children and adolescents from this population as well (Frisbee et al. 2010). Additionally, decreased serum triiodothyronine levels and elevated thyroxine levels (Knox et al. 2011) and

elevated alanine aminotransferase levels following PFOA exposure were detected in human samples (Gallo et al. 2012). Associations between PFOA exposure and testicular, kidney, prostate, and ovarian cancers and non-Hodgkin lymphoma were also reported (Vieira et al. 2013). Conversely, Nolan et al. (2009; 2010) reported that elevated PFOA exposure in this population was not associated with lowered birth weight or gestational age, labor or delivery complications, or maternal risk factors such as eclampsia or diabetes. However, lack of umbilical cord serum PFOA concentrations made it difficult to assess actual fetal exposures and more work is needed before conclusions can be made regarding the potential negative health effects of PFOA in this human population. In a recent study of the Faroe Islands population, developmental exposure to a mixture of perfluorinated compounds (PFCs) was associated with decreased humoral immune responses to vaccinations in children between five and seven years old. (Grandjean et al. 2012).

In laboratory animal models, PFOA is known to activate PPARα to induce hepatic, pancreatic, and testicular cell cancers (Biegel et al. 2001), endocrine disruption (Olsen et al. 1998), and immunotoxicity (DeWitt et al. 2008). In addition, PFOA has been reported to induce developmental effects, including retarded development and decreased fetal survival (Wolf et al. 2007). An important reason for this study is that the precise mechanisms of developmental toxicity for PFOA have not yet been fully elucidated.

1.5 Heart development

The heart is one of the earliest organs to develop and become functional during the embryonic stage; proper circulation is a prerequisite for many other organs to properly develop **(**Farrell and Kirby 2001**)**. The heart is composed of cells derived from several different origins,

and yet these cells form a morphologically and functionally refined organ in a relatively short time (Farrell and Kirby 2001; Kirby 2002**)**. In chickens, the primary heart tube starts to develop within hours of incubation, twists into the beginnings of four chambers by day four and is fully four chambered by day seven (Farrell and Kirby 2001; Kirby 2002**)**. In a very short time window, many signals regulate heart development, which makes it vulnerable to exogenous disruptions that may induce abnormalities of form or function. Because heart development happens in such a short time, the window during which exposure to an exogenous disruption occurs is a crucial factor in developmental cardiotoxicity. Signaling pathways important in regulating heart development and that may be impacted by developmental PFOA exposure include TGF-β superfamilies, transcription factor nkx2.5 and GATA4 (Farrell and Kirby 2001; Kirby 2002).

1.5.1 TGF-β superfamilies in heart development

Certain members of the TGF-β superfamilies, including BMP2, BMP4, TGF-β, and activin play important roles in early heart development, especially in heart field mesoderm induction **(**Kirby 2002). BMP2 is expressed in the anterior lateral endoderm, which is the crucial induction center for heart field formation (Ladd et al. 1998). In the precardiac mesoderm, application of BMP2 in vivo induces the expression of cardiac specific transcription factors Nkx 2.5 and GATA4, whereas application of the BMP2 antagonist noggin blocks cardiac induction (Schultheiss et al. 1997; Schneider et al. 2001). Upon binding to BMP2, BMP2 receptors undergo autophosphorylation, and then phosphorylate the second messenger SMAD1/5/8, which then forms a heteromeric complex with SMAD4 and transfers to the nucleus to regulate gene expression (Chen et al. 2004). Figure 1.1 contains a schematic of the BMP2 signaling pathway.

1.5.2 Nkx2.5 and GATA4 in heart development

Nkx2.5 is a homeodomain protein that acts as a transcription factor and regulates many downstream genes of cardiac development, including Lrrn1, Elovl2, Safb, Slc39a6, Khdrbs1, Hoxb4, Fez1, Ccdc117, Jarid2, Nrcam, and Enpp3 (Barth et al. 2010). Thus Nkx2.5 regulates many aspects of cardiac development. In the early stage of heart development, Nkx2.5 plays an important role in cardiac precursor differentiation and proliferation (Harvey et al. 2002; Kirby 2002). Later developmental events such as outflow tract formation also require Nkx2.5 (Harvey et al. 2002). Loss of Nkx2.5 leads to failed cardiac looping and deficient myocardial differentiation, which then leads to lethality in mouse embryos (Tanaka et al. 1999). GATA4 is a zinc finger transcription factor that also plays a crucial role in cardiac development (Peterkin et al. 2005). Examples of the target genes of GATA4 include LHX9, BCL2 and N-cadherin (Smagulova et al. 2008; Kobayashi et al. 2006; Zhang et al. 2003). Lack of GATA4 expression leads to abnormal folding, failure of heart primordial fusion, and dendoderm defects, which also lead to lethality in mouse embryos (Kuo et al. 1997; Molkentin et al. 1997). Nkx 2.5 and GATA4 could regulate each other's expression (Sepulveda et al. 2002). Additionally, Nkx2.5 is regulated by the BMP2 pathway (Prall et al. 2007), thus BMP2, Nkx2.5, and GATA4 are closely connected in their regulation of heart development.

1.6 Heart development and environmental contaminants

Heart development is a complex process involving many signaling pathways that regulate development in a very short time. This makes heart development especially sensitive to exogenous disruptions. When environmental contaminants mimic or block endogenous signals and/or ligands, or indirectly alter signaling pathways, for example, by changing phosphorylation

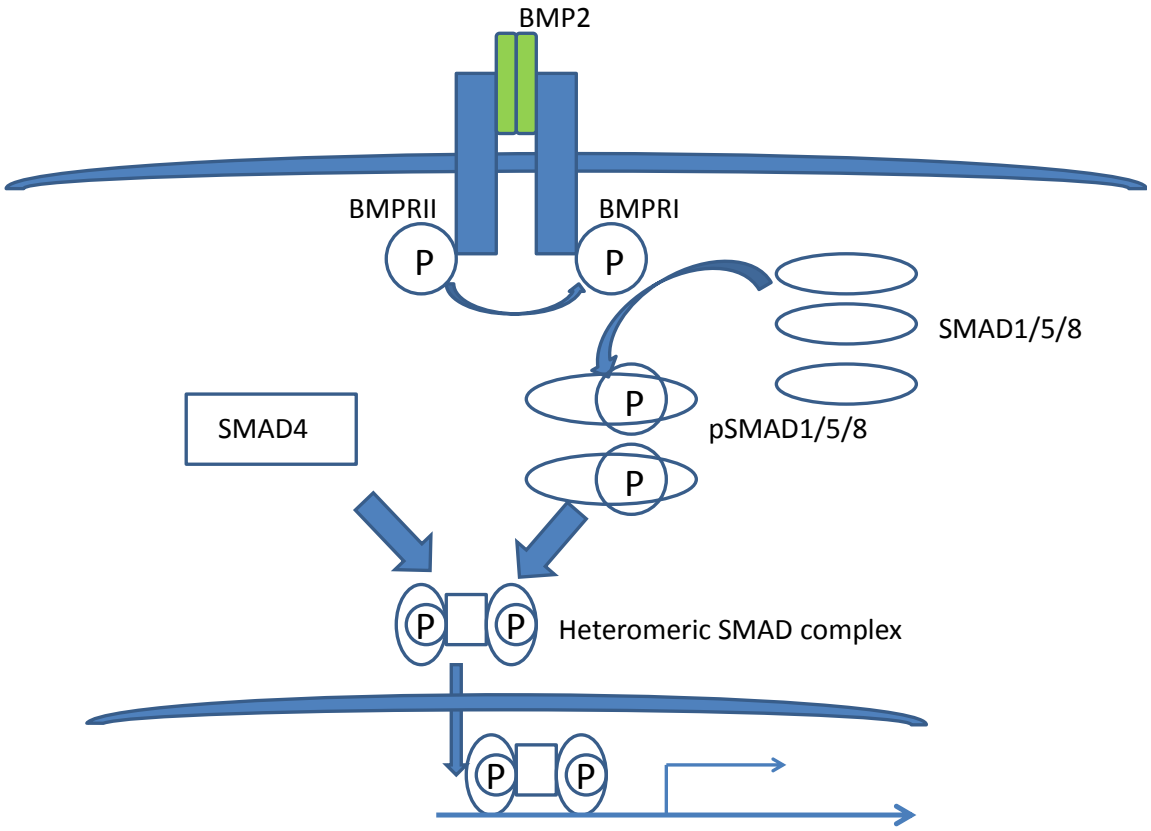


Figure 1.1 BMP2 signaling pathway. When BMP2 ligands bind to the receptor, BMP receptor type II phosphorylates BMP receptor type I, which then phosphorylates the second messenger SMAD1/5/8, and forms the heteromeric SMAD complex with SMAD4. The complex translocates to the nucleus and acts as a transcription factor, regulating the expression of important heart development-related genes, such as Nkx2.5 and GATA4.

states when the heart is developing, the risk of malformations increases. Major malformations will lead to congenital heart diseases (CHD). CHD is defined as defects present in the structure of the heart and/or great vessels at birth (Go et al. 2013), and is the leading cause of infant death, accounting for 29% of total infant mortality in the U.S. (Go et al. 2013). 36,000 infants are born in the U.S. with congenital heart disease every year (Go et al. 2013). Major types of congenital heart disease include visceroatrial heterotaxia or right–left axis defects, alignment defects such as dextroposed aorta and tricuspid atresia; septation defects such as atrioventricular canal, ventricular septal defects, persistent truncus arteriosus, aortic stenosis and interrupted aortic arch (Kirby 2002).

Examples of agents that induce developmental cardiotoxicity include: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which inhibits epicardium and proepicardium development in zebra fish (Plavicki et al. 2012); trichloroethylene alters shear stress gene expression and function in developing chicken hearts (Makwana et al. 2010); and finally, PFOS, a PFC that shares some properties with PFOA, can impair the cardiac development of marine medaka (Huang et al. 2011). Aside from agents that can induce specific types of developmental cardiotoxicity, environmental toxicants also may induce developmental cardiotoxicity via indirect mechanisms, such as interference with energy metabolism (Hantson et al. 2012). More attention should be paid to the potential risks that environmental contaminants pose on developing organs, including the heart, because of the wide range of environmental contaminants and the high human health risk associated with disrupted organ development, including heart development.

1.7 PFOA and heart development

Presently, no association has been reported between developmental exposure to PFOA and an increased incidence of CHD in human populations. However, it is never easy to establish associations between exposure to a single chemical and the etiology of a specific disease. Associations, if present, between PFOA and CHD will likely be confounded by other risk factors, including exposure to multiple chemicals. Because PFOA does induce a wide range of toxicities, including immunotoxicity, developmental toxicity, and disturbances to energy metabolism, it may induce CHD through multi-system toxicity. However, additional investigations are still necessary to determine whether exposure to PFOA (and other PFCs) contributes to the etiology of CHD.

1.8 Heart development and cardiovascular diseases

The etiology of cardiovascular disease is complex. During 2009, over 2150 Americans died of cardiovascular disease every day and every one in six deaths in the US in 2009 was due to cardiovascular diseases (Go et al. 2013). Given that cardiovascular disease is the leading cause of morbidity and mortality in the US, identification of potential risk factors can help to reduce the disease burden on society. Many risk factors such as smoking and diet have been identified, but many more remain unknown. Disruption of heart development during the embryonic stage by exogenous agents could be a risk factor. While profound disruption of heart development likely will cause congenital heart disease, minor disturbances that have no detectable effects early in life could lead to a higher heart disease incidence later in life (Langley-Evans et al. 2006; Eriksson et al. 2006). If we can identify these disturbances, such as exposure to environmental contaminants, it is possible to take measures to decrease the incidence of cardiovascular disease.

1.9 Chicken embryo models in developmental toxicity studies

The history of using chicken embryos as a model in developmental toxicology is very long. In the early nineteenth century, Etienne Geoffroy Saint-Hilaire had already started using chicken embryos to study the effects of various environmental conditions on development (Charon. 2004). There are several advantages to using chicken embryos in developmental toxicological studies. Eggs are laid before major development begins, so in ovo development is relatively free of maternal influences. Because of the long history of using chicken embryos, the embryonic development of chickens has been very intensely studied and well defined, making it very easy to identify abnormalities associated with exposure to toxicants. It is also possible to isolate embryonic tissues at specific ages relatively easily (Henshel et al. 2003). In the current study, hearts from embryonic day 19 (ED19) chickens were used for histology as they are the size of hatchling chickens but have not yet undergone the highly energetic process of hatching and thus are slightly less variable. ED4 embryos were easily collected from eggs and were ideal tissues for PCR and western blot. Under a dissection microscope, hearts of ED6 embryos were dissected so that expression of heart-specific genes and protein levels could be measured expressly in hearts. The chicken embryo model facilitated this dissertation work and has proved to be especially useful in developmental studies.

1.10 Rationale of investigating developmental cardiotoxicity induced by PFOA

Although much work has been done regarding developmental toxicity induced by PFOA, the mechanisms of retarded development and decreased fetal survival have not yet been elucidated. I hypothesize that as the cardiovascular system is one of the earliest organ systems to develop, cardiotoxicity induced by developmental PFOA exposure may contribute to decreased

fetal survival. Moreover, published data suggest that PFOA has the potential to interfere with heart development. Possible molecular targets of PFOA that could induce developmental cardiotoxicity are: PPARα, inflammatory cytokines, and the BMP2 signaling pathway.

1.10.1 PPARα

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are capable of producing hepatic peroxisome proliferation on activation (Dreyer et al. 1992). Upon activation, PPARs heterodimerize with the retinoid X receptor and bind to the promoter region of target genes controlling fatty acid uptake, utilization, oxidation, and storage pathways (Feige et al. 2006). The PPAR family includes PPARα, PPARβ/δ and PPARγ (Dreyer et al. 1992). Since PFOA is known to activate PPARα (Rosen et al. 2008) and PPARα is expressed in relatively abundant levels in the heart (Lee et al. 2013), PPARα is one of the most probable targets of PFOA in developmental cardiotoxicity. To date, no published data indicate that PPARα is directly involved in heart development. However, PPARα is known to regulate energy metabolism (Nicholls et al. 2012). PPARα overexpression could lead to a cardiac phenotype similar to that observed in hearts of diabetics (Finck et al. 2002). Considering the critical role of PPARα in modulating fatty acid β-oxidation, the major energy resource of the heart, it is possible that PPARα is involved in developmental cardiotoxicity via energy metabolism modulation. Additionally, PPARα activation is associated with anti-inflammatory and anti-oxidant effects (Chen et al. 2007); PPARα could affect heart development via altered ROS generation as well. ROS generation is one of the major factors that mediate cardiotoxicity **(**Berthiaume and Wallace 2007). While PPARα activation has been reported as a protective factor against ROS in myocardiac diseases (Ibarra-Lara et al. 2013), Sambandam et al. (2005) reported that chronic activation of PPARα is detrimental to ischemic hearts. In an early clinical trial, increased overall

mortality was observed in subjects who took the PPARα agonist clofibrate and who also had a history of myocardial diseases (Backes et al. 2007). These reports demonstrate the potential of PPARα activation to induce detrimental effects in the heart, possibly via excessive fatty acid β-oxidation followed by ROS generation. In a developing chicken embryo, β-oxidation of fatty acids derived from yolk lipids is the major energy resource (Stock and Metcalfe 1987). These higher basal levels of β-oxidation of fatty acids and relatively high vulnerability of the developing embryo to ROS generation (Surai 1999) suggest that PPARα may partially mediate developmental cardiotoxicity induced by PFOA exposure. In summary, interference of PPARα signaling by PFOA could potentially affect heart development via mechanisms such as alteration of energy metabolism and excessive ROS generation.

1.10.2 Inflammatory cytokines

Inflammatory cytokines play an important role in cardiotoxicity (Torre-Amione 2005). The induction/suppression of inflammatory cytokine production could potentially contribute to the developmental cardiotoxicity that we observed with PFOA exposure, as cytokines could interfere with signaling pathways that are important to heart development. For example, the TGF-β superfamily is vulnerable to interference by inflammatory cytokines. IL-1β can modulate TGF-β signaling via IL-6 activation (Luo et al. 2009) and increased TNF-α can suppress the TGF-β receptor population (Yamane et al. 2003). The effects of PFOA exposure on inflammatory cytokines suggest both pro- and anti-inflammatory effects. While Taylor et al. (2005) reported that PFOA has potency similar to dexamethasone, a known anti-inflammatory agent, against carageenan-induced edema and thermal hypersensitivity, Yang (2010) reported that PFOA exposure increased expression of various inflammatory cytokines in *Oryzias latipes*. In this dissertation, the impact of PFOA on inflammatory cytokines in chicken embryos was

assessed to elucidate the role of inflammatory cytokines in PFOA-induced developmental cardiotoxicity.

1.10.3 BMP2 signaling pathway

The BMP2 signaling pathway belongs to the TGF-β superfamily. As detailed in 1.5.1, the BMP2 signaling pathway plays important roles in heart development. Not only is BMP2 itself crucial for heart field induction, but the downstream genes regulated by BMP2 also include the heart-development-important transcription factors Nkx2.5 and GATA4 (Kirby 2002). Furthermore, as mentioned in 1.10.2, there is evidence that PFOA could indirectly affect the BMP2 signaling pathway by interfering with inflammatory cytokines. The ability of PFOA to interfere with the BMP2 ligand, receptor, and second messengers was assessed as a potential mechanism of toxicity for PFOA induced developmental cardiotoxicity.

1.11 Aims of the study

The aim of this study was to assess the developmental cardiotoxicity induced by PFOA exposure in chicken embryos and hatchling chickens and to explore the underlying mechanisms. Specifically, there were three aims of the study:

Specific aim 1: To assess the morphological and functional alterations in hearts from late stage chicken embryos and hatchling chickens following developmental exposure to PFOA.

Rationale for specific aim 1: It is most important to first confirm that PFOA does induce developmental cardiotoxicity. This was the basis for all of the subsequent studies. To evaluate cardiotoxicity, morphological and functional parameters were endpoints most relevant to human health. These results not only indicated cardiotoxicity, but also provided hints of the potential mechanisms.

Specific aim 2: To assess the molecular mechanism of PFOA-induced developmental cardiotoxicity, including PPARα, the BMP2 pathway, Nkx2.5/GATA4, and inflammatory cytokines.

Rationale for specific aim 2: Determining the molecular mechanism of toxicity is necessary to fully understand how PFOA disrupts cardiac development. As PFOA is known to activate PPARα, signals associated with the PPARα pathway were assessed as the most plausible mechanism. PFOA also is known to interfere with inflammatory cytokines; the latter potentially can interfere with the BMP2 signaling pathway, so both inflammatory cytokines and targets within the BMP2 signaling pathway were assessed as well. Moreover, as the BMP2 signaling pathway is known to induce Nkx2.5 and GATA4 expression, both of which are important transcription factors in heart development, they also were assessed.

Specific aim 3: To assess PFOA-induced developmental cardiotoxicity at the cellular level, including viability, contractility, and ROS generation.

Rationale for specific aim 3: While a mechanism of PFOA-induced developmental cardiotoxicity was suggested by data generated in specific aim 2, others may exist and still remain unknown. This aim was designed to discover additional information regarding the mechanism of cardiotoxicity, mainly at the cellular level. This aim asked whether PFOA induced developmental cardiotoxicity via overt cytotoxicity of PFOA to cardiomyocytes or via an earlier, upstream signaling pathway. To answer this, primary cardiomyocyte cultures isolated from hearts of chicken embryos pre-exposed to PFOA (exposed to PFOA in ovo) or exposed to PFOA in vitro (directly exposed to PFOA in medium) were assessed and the responses were compared to distinguish whether PFOA impacted cells directly or by disturbing a process during development.

CHAPTER 2 PERFLUROOCTANOIC ACID INDUCES DEVELOPMENTAL CARDIOTOXICITY IN CHICKEN EMBRYOS AND HATCHLINGS

Abstract

Perfluorooctanoic acid (PFOA) is a widespread environmental contaminant that is detectable in serum of the general U.S. population. PFOA is a known developmental toxicant that induces mortality in mammalian embryos and is thought to induce toxicity via interaction with the peroxisome proliferator activated receptor α (PPARα). As the cardiovascular system is crucial for embryonic survival, PFOA-induced effects on the heart may partially explain embryonic mortality. To assess impacts of PFOA exposure on the developing heart in an avian model, we used histopathology and immunohistochemical staining for myosin to assess morphological alterations in 19-day-old chicken embryo hearts after PFOA exposure. Additionally, echocardiography and cardiac myofibril ATPase activity assays were used to assess functional alterations in one-day-old hatchling chickens following developmental PFOA exposure. Overall thinning and thinning of a dense layer of myosin in the right ventricular wall were observed in PFOA-exposed chicken embryo hearts. Alteration of multiple cardiac structural and functional parameters, including left ventricular wall thickness, left ventricular volume, heart rate, stroke volume, and ejection fraction were detected with echocardiography in the exposed hatchling chickens. Assessment of ATPase activity indicated that cardiac myofibril calcium independent ATPase activity also was elevated by developmental PFOA exposure. In summary, the heart appears to be a developmental target of PFOA. Additional studies will investigate mechanisms of PFOA-induced developmental cardiotoxicity.

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2.1 Introduction

Perfluoroalkyl acids (PFAAs) are fluorinated compounds used to manufacture materials for myriad consumer and industrial products, including nonstick, stain-repellant, water repellant, and fire-retardant coatings. Perfluorooctanoic acid (PFOA) is a PFAA that is a polymerization aid used to manufacture fluorinated polymers and elastomers, the most well known being polytetrafluoroethylene (PFTE). According to the current major manufacturer of PFOA, a minimal amount of PFOA is still present in end-products (DuPont 2011), which can leach out and may contribute to exposures. PFOA also is a breakdown product of certain fluorinated telomer alcohols and other precursor compounds of fluorinated polymers (Wang et al. 2005). PFOA has become a public health concern because it is present in environmental media and biota.

Increasing reports of PFOA-induced toxicity led to a stewardship program between major flourochemical manufacturers and the U.S. Environmental Protection Agency (USEPA). The goal of the stewardship program is to eliminate PFOA and precursor products that can break down to PFOA by 2015 (USEPA 2011). However, as PFOA does not bio-degrade, it persists in the environment; it is ubiquitously found in environmental samples and in serum of the world population. The median serum concentration in the U.S. population reported in 2007-2008 was 4.3 ng/mL (CDC 2011). In an area of the Mid-Ohio Valley contaminated with PFOA by a manufacturing plant, epidemiological studies of exposed populations reported a median PFOA serum concentration of 26.6 ng/mL, with a high value of 17,556.6 ng/mL (Steenland et al. 2009). Epidemiological studies of the effects of PFOA on human embryonic and fetal development of residents living in the Mid-Ohio Valley have not produced clear associations with birth outcomes. Stein et al. (2009) reported modest association of PFOA serum concentrations with

preeclampsia and birth defects in the Mid-Ohio Valley population. Additional studies reported that elevated PFOA exposure in this population was not associated with increased risk of lowered birth rate or gestational age (Nolan et al. 2009) or with congenital anomalies, labor or delivery complications, or maternal risk factors such as eclampsia or diabetes (Nolan et al. 2010). Although Apelberg et al. (2007) reported PFOA concentrations in umbilical cord serum for the general U.S. population (0.3-7.1 ng/mL), Nolan et al. (2009, 2010) did not measure PFOA concentrations in umbilical cord serum, so no corresponding cord serum levels are available for the highly exposed Mid-Ohio Valley U.S. population. Regardless, given the PFOA serum concentrations reported for this population and the potential for PFOA to affect development, additional studies of more subtle developmental effects are necessary.

In laboratory models, PFOA induces multisystem and data from studies of such models suggest that it is an agonist of the peroxisome proliferator activated receptor α (PPARa). Effects in laboratory models include hepatic, pancreatic and testicular cancer (Biegel et al. 2001), endocrine disruption (Olsen et al. 1998), and immunotoxicity (DeWitt et al. 2008). In addition, PFOA has been reported to induce developmental effects, including retarded development, decreased fetal survival and increased deformities in animal models (Wolf et al. 2007). In humans, epidemiology studies indicate that PFOA exposure is associated with elevated serum cholesterol and uric acid levels (Steenland et al. 2010).

Measurable serum concentrations and accompanying changes in cholesterol levels in exposed human populations warrant investigation of additional health effects of PFOA, especially effects related to the cardiovascular system. In addition, reasons for increases in fetal mortality associated with developmental PFOA exposure (Wolf et al. 2007; Lau et al. 2004, and DeWitt et al. 2009) are unknown. As cardiovascular development is a complex and delicate

process, with numerous signaling activities vulnerable to exogenous disruptions from exposure to pathogens, drugs and environmental pollutants, altered cardiovascular development may affect embryo survival. Several agents have been reported to alter cardiovascular development. Lipopolysaccharide exposure induces cellular hypertrophy in H9c2 myocardiac cells and alters the calcineurin/NFAT-3 signaling pathway (Liu et al. 2008). Adriamycin (doxorubicin), an antitumor drug used to treat leukemias, lymphomas and neoplasms, induces ventricular septal defects, dextroposition of the aorta, and aortic arch anomalies in a dose-related manner when topically administered to five-day-old chicken embryos (Takaqi et al. 1989). Adriamycin likely decreased embryonic cardiac blood flow and inhibited rapidly exchangeable calcium within cardiac cells (Takaqi et al. 1989). In addition, adriamycin induces dose-dependent cardiomyopathy, likely via iron oxidation and oxygen free radical formation, which limits its therapeutic applications (Shi et al. 2011). Environmental contaminants also have been reported to induce cardiotoxicity. 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) application enlarged left and right ventricles, thickened ventricular septa and thinned left ventricle walls in chicken embryos (Walker et al. 1997) and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) exposure decreased myocyte proliferation in zebra fish, possibly by affecting hemodynamics (Grimes et al. 2008). Taken together, these studies indicate that the developing heart is sensitive to exogenous perturbations. Given PFOA's developmental toxicity in laboratory models, presence in humans and wildlife, and possible effects on endogenous compounds associated with heart disease, we investigated PFOA's effects on developing hearts. As avian and mammalian cardiovascular development is similar and avians lack a direct maternal influence, avian embryos are ideal models for developmental toxicity studies. This is the first study to assess both morphological and functional changes in avian hearts developmentally exposed to PFOA.

2.2 Materials and methods

2.2.1 Animals

Fertile chicken (*Gallus gallus*) eggs were purchased from North Carolina State University Poultry Research Center (Raleigh, NC). Eggs were cleaned with 20% povidone iodine, candled to outline air cells in pencil on shells, weighed, given ID numbers, and evenly distributed by weight among doses. Due to incubator space limitations, eggs were incubated in eight batches of 40-58. Uninjected eggs were included in each batch as environmental controls.

2.2.2 Chemicals

Sunflower oil was purchased from Spectrum Organic Products, LLC (Boulder, CO). PFOA and other chemicals (if not otherwise mentioned) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.3 Egg injection and incubation

Injection procedures were as described in Henshel et al. (2003). Briefly, PFOA was suspended in sunflower oil and vortexed before injection into each egg. An awl was used to drill a 1 millimeter (mm) hole into the middle of air cells and PFOA doses of 0, 0.5, 1 and 2 milligram per kilogram (mg/kg) of egg weight were injected with a gel-loading pipette tip. Stock solutions were prepared so that 0.1 microliter (µl) of solution per gram of egg weight resulted in the appropriate mg/kg concentration. Doses were chosen based on published reports of PFAAs injected into chicken eggs and were not expected to induce dose-related mortality. After injection, a drop of melted paraffin sealed injection holes. Eggs were incubated in a Lyon Roll-X incubator (Chula Vista, CA) set at 99.5-100ºF dry bulb and 87-88ºF wet bulb. Eggs were candled every 2-3 days; infertile/undeveloped/dead eggs were removed. After external pipping eggs were placed individually into containers large enough for the hatched chickens and

transferred to a larger incubator (G.Q.F. Manufacturing Co., Savannah, GA). Hatchling chickens were kept in a warmed brood box until euthanasia. All procedures were approved by the East Carolina University Institutional Animal Care and Use Committee.

2.2.4 Histology

At embryonic day 19 (D19; two days prior to hatch), embryos were removed from eggs and quickly decapitated. Whole embryo, yolk, heart, and liver weights were recorded. Hearts were excised, rinsed in ice cold saline to fully dilate ventricles, fixed in 10% phosphate buffered formalin for 24h, and cut transversely. Cuts were made approximately 60% of the length of the heart from the ventricular apex (Figure 2.1A). Ventricular tissues were processed routinely (Thermo Scientific Shandon Citadel 1000, Waltham, MA), embedded in paraffin, and sliced at six micrometer (μm) per section on a rotary microtome (Thermo Scientific, HM 315 Waltham, MA). The septomarginal trabecula was used to maintain a relatively constant position to ensure that ventricular wall measurements were made at the same location within each heart (Figure 2.1D-F). For routine histology, hearts were stained with Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) and Eosin Y solution (Harleco, Gibbstown, NJ) (H&E). Adobe Photoshop (San Jose, CA) and SPOT advance (SPOT imaging, Sterling Heights, MI) with rulers (made in Berkeley Logo, Berkeley, CA) was used to measure the thickness of the right ventricular wall (Figure 2.1B). Average right ventricular wall thickness was normalized to whole heart weight to minimize potential PFOA-induced effects on body weight or developmental stage.

2.2.5 Immunohistochemistry

Immunohistochemistry was used to selectively stain sarcomere myosin. Antigen Unmask Solution, Vectastain ABC Kit (Mouse IgG), and peroxidase substrate Kit were purchased from

Vector Labs (Burlingame, CA); MF-20 antibody against myosin was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa city, IA). Sections were deparaffinized, blocked in serum albumin for one hour, and incubated with 1:50-1:100 MF-20 in phosphate buffered saline with 0.5% tween-20 for 30 minutes at room temperature. Secondary antibody was applied at 1:75 and 3,3'-Diaminobenzidine (DAB) with nickel solution was added for color development. Sections were counter-stained with hematoxylin for 20 seconds and cover slipped. Although myosin is present in all myocytes, a dense layer of myosin selectively stained in the right ventricular wall was used as the measurement target (Figure 2.1C). Measurements similar to those on H&E stained sections were collected, with an additional measure to evaluate the myosin dense layer, which also was normalized to whole heart weight.

2.2.6 Cardiac ultrasound on hatchling chickens

Within 24 hours post-hatch, hatchlings from all dose groups (including the vehicle control group) were evaluated with an ultrasound instrument (Visualsonics Vevo 2100, Toronto, Ontario, Canada) for direct cardiac function measurement. Ultrasound measurements were collected from hatchlings in random order to spread time and handling-related differences across dose groups. Hatchlings were enclosed in a stockinette (stretchable, breathable gauze) so that they could be restrained for echocardiography without damaging their feathers. A hole was cut in the torso of the stockinette and ultrasound gel was applied directly to the torso of each hatchling. Heart rate, stroke volume, ejection fraction (fraction of blood pumped out of the right and left ventricles with each heart beat), fraction shortening (the fraction of any diastolic dimension that is lost in systole), left ventricular posterior wall dimension, left ventricular volume and mass were measured and analyzed; the person performing the analysis was blind to the dose group assignments. Although histology was evaluated on the right ventricular wall, the left ventricle is

a more accessible target for echocardiography. While we are aware of the potential limitation of taking histological and functional measurements on different ventricles, the closely related functions of the ventricles enable us to evaluate the effects of PFOA exposure on overall heart development.

2.2.7 Cardiac myofibril ATPase assay

To supplement the ultrasound data and provide an additional functional measure, animals were euthanized shortly after ultrasound measurements and a cardiac myofibril ATPase assay was used to determine if calcium–activated, magnesium-dependent ATPase activity was altered. The method used was adapted from Candau et al. (2003) with modifications. Hearts were removed as described for the morphology study and stored at -80ºC. Thawed ventricular tissue (200 milligram (mg) per animal) was homogenized in 1 milliliter (mL) homogenization buffer, which consisted of tris (hydroxymethyl) aminomethane (Tris) 50 millimolar (mM), ethylenediaminetetraacetic acid (EDTA) 5mM, sodium chloride (NaCl) 100mM, potassium chloride (KCl) 2mM, 1:100 protease inhibitor (Thermal Scientific, Rockford, IL), 0.5% Triton, pH 7.2-7.4 and then centrifuged at 2000 relative centrifugal force (RCF, g) (4ºC, 15 minutes). Pellets were resuspended in 1 mL wash buffer (KCl 100mM and 1:100 protease inhibitor) and centrifuged at 2000g (4ºC, 15 minutes). Washes were repeated four times. Prior to the last centrifugation, suspensions were filtered through 70 µm nylon cell strainers (BD Falcon, Bedford, MA) to exclude tissue debris. Final pellets were resuspended in storage buffer (Tris 50mM, potassium acetate (K-acetate) 100mM, KCl 5mM and 1:100 protease inhibitor, pH 7.2-7.4) and protein concentration was determined (Thermo Scientific, Rockford, IL).

Protein concentration was adjusted to 50 microgram per milliliter (ug/mL), and samples were divided into calcium-dependent ATPase groups (storage buffer plus 3mM magnesium

chloride and 0.1mM calcium chloride) and calcium-independent ATPase groups (storage buffer plus 3mM magnesium chloride and 10mM ethylene glycol tetraacetic acid (EGTA)). Samples were equilibrated at room temperature for 5 minutes and ATP (final concentration 0.5mM) was added to initiate reactions. After 30 minutes at room temperature, PiGoldLock (Innova Biosciences, Babraham, Cambridge, UK) with 1:100 accelerator was added to stop reactions and visualize free inorganic phosphate. Two minutes later, stabilizer (Innova Biosciences, Babraham, Cambridge, UK) was added to stop ATP hydrolysis. Samples were incubated at room temperature for 30 minutes to develop color completely and read at 630 nanometer (nm) (Biotek Synergy HT plate reader, Winooski, VT).

2.2.8 Serum PFOA concentration

Samples were prepared as described in Reiner et al. (2009). Briefly, thawed serum was vortexed for 30 seconds and 25 microliter (µl) aliquots were placed into 5 mL polypropylene tubes (BD Falcon, Franklin Lakes, NJ) and denatured with 100 µl of 0.1 molar (M) formic acid containing approximately 5 ng $^{13}C_2$-PFOA. Samples were vortexed for 1 minute (min), received 1.0 mL of cold acetonitrile to precipitate proteins, were vortexed for 1 min, and then centrifuged at 10,000 revolutions per minute (RPM) for 2 min to pelletize proteins. Aliquots of extracts (200 ul) were combined with 200 µl of 2 mM ammonium acetate for liquid chromatography–mass spectrometry (LC/MS-MS) analysis. All unknowns, replicates, method and matrix blanks, and quality control (QA/QC) samples were prepared in this fashion. Standard curve preparation was matrix matched. Standards were prepared by adding 25 µl of control Pel-Freez CD1 mouse serum into a 5 mL tube and then spiking in a corresponding mass of PFOA in methanol (0.250 – 1,250 ng) relating to 10 to 50,000 ng PFOA/mL of serum. This was covered by two standard curve ranges (10 – 500 ng/mL) and (500 – 50,000 ng/mL). Standards were likewise treated as all

other samples. Samples that did not fall in the lower standard curve range were re-run with a slightly modified method. The 25 µl serum sample was diluted with 2.0 mL of 0.1 M formic acid, 200 µl was sampled and was added to 2.0 mL of acetonitrile containing approximately 62.5 ng of $^{13}C_2$-PFOA. This acetonitrile extract was then used to prepare the samples further as described above.

Samples were quantitated using a Waters Acquity UPLC coupled to a Quatro Premier XE tandem mass spectrometry (MS/MS) (Waters, Milford, MA). Samples were run in a batch to include double blanks (solvent blank), a method blank, matrix blank (blank serum), standards, QC samples, replicates, and unknowns in sequence. Standards were run at the beginning and end of analytical batches, and QC samples interspersed in analytical batches. Quality control samples consisted of pooled mouse serum spiked at six concentrations over the analytical range. Average accuracy of QC pools samples was 94.1% with a %RSD of less than 10% for all samples (n = 18). PFOA was monitored via the transition 413-369 and 413-169 and for the $^{13}C_2$-PFOA 415-370. Samples were run using an isocratic (50:50) mobile phase consisting of 2 mM ammonium acetate aqueous solution with 5% methanol (solvent A) and 2 mM ammonium acetate in acetonitrile (solvent B). Samples were integrated using the equipment software and corrected if necessary by the operator.

2.2.9 Statistical Analysis

All data were analyzed by one way Analysis of variance (ANOVA) by dose. Statistical significance was determined when $P < 0.05$. When ANOVA revealed a statistically significant model, post-hoc t-tests were performed to determine statistical significance between dose groups.

2.3 Results

2.3.1 General toxicity

Mortality, hatchability, yolk-free body weight, and relative heart and liver weights for both D19 chicken embryos and hatchling chickens are reported in Table 2.1. In D19 embryos, mortality in the 2 mg/kg dose group was statistically increased by 75.7% relative to the vehicle control group. No other differences in general toxicity parameters were observed in D19 embryos. In hatchling chickens, relative liver weight was statistically increased by 28.6% in the 0.5 mg/kg and 1 mg/kg dose groups relative to the vehicle control group. No other differences in general toxicity parameters were observed in hatchling chickens.

2.3.2 Cardiac morphology

Right ventricular wall thickness and a myosin dense layer thickness were evaluated with H&E staining and immunohistochemistry for sarcomere myosin. Thinning of the right ventricular wall (corrected for whole heart weight) was observed in H&E stained tissues from the 1 mg/kg and 2 mg/kg dose groups relative to vehicle controls (Figure 2.2A). The 1 mg/kg and 2 mg/kg dose groups were 20.2% and 22.7% thinner, respectively, relative to vehicle controls ($P < 0.05$). Staining of the myosin dense layer revealed thinning of the myosin dense layer in the right ventricular wall in the 1 mg/kg and 2 mg/kg dose groups relative to vehicle controls (Figure 2.2B). The 1 mg/kg and 2 mg/kg groups were 27.1% and 28.6% thinner, respectively, relative to vehicle controls ($P < 0.05$).

2.3.3 Cardiac ultrasound

All morphological parameters evaluated by cardiac ultrasound demonstrated statistical significance relative to vehicle controls. The left ventricular posterior wall dimension at diastole (Figure 2.3A) increased by 5.7% (0.5 mg/kg), 29.6% (1 mg/kg), and 71.6% (2 mg/kg). The left

ventricular posterior wall dimension at systole (Figure 2.3B) was increased 4.9% (0.5 mg/kg), 11.5% (1 mg/kg), and 26.0% (2 mg/kg). Left ventricular volume at diastole (Figure 2.3C) was increased by 6.7% (0.5 mg/kg) and decreased by 14.1% (1 mg/kg) and 40.4% (2 mg/kg). At systole, left ventricular volume (Figure 2.3D) was decreased by 12.2% (0.5 mg/kg), 48.5% (1 mg/kg), and 76.0% (2 mg/kg). Finally, left ventricle mass (Figure 2.3E) was increased by 5.4% (0.5 mg/kg), 8.8% (1 mg/kg), and 24.7% (2 mg/kg). Left ventricular mass was the only morphological parameter that differed statistically between uninjected controls and vehicle controls; the mass of the vehicle controls was 5.1% greater than in uninjected controls. No other morphological differences were observed between uninjected controls and vehicle controls.

Functional parameters collected by cardiac ultrasound also were statistically significant. Heart rate (Figure 2.4A) was decreased by 7.9% (0.5 mg/kg) and increased by 55.3% (1 mg/kg) and 52.9% (2 mg/kg). Stroke volume (Figure 2.4B) was elevated in the 0.5 mg/kg group (12.6%) and decreased in the 1 mg/kg group (3.4%) and 2 mg/kg group (29.5%). The ejection fraction (Figure 2.4C) was elevated by 5.4% (0.5 mg/kg), 9.0% (1 mg/kg), and 17.0% (2 mg/kg). Fraction shortening (Figure 2.4D) was elevated by 6.3% (0.5 mg/kg), 12.6% (1 mg/kg), and 30.0% (2 mg/kg). No functional differences were observed between uninjected controls and vehicle controls.

2.3.4 Cardiac myofibril ATPase

An increased ratio of calcium-independent ATPase activity to calcium-dependent ATPase activity was observed, but the difference was not statistically significant (Figure 2.5).

2.3.5 Serum concentration of PFOA

Mean serum concentrations of PFOA in vehicle controls and uninjected controls were below limits of quantitation (LOQ) of 50 ng/mL. Mean PFOA serum concentrations in dosed

hatchlings (mean ± standard deviation) were 1230.8 ± 363.9, 2055.7 ± 1577.5, and 5670.0 ± 3394.1 ng/mL for the 0.5, 1, and 2 mg/kg dose groups, respectively (Figure 2.6). Quality control standards were all within acceptable parameters, with coefficients of variation of less than 5%.

2.4 Discussion

From our observations, developmental exposure to PFOA in an avian model leads to alteration of heart morphology and function, which are effects similar to those observed in human congenital heart diseases as well as generalized cardiovascular diseases. Cardiac alterations were observed at doses that did not increase mortality or other markers of generalized toxicity in either D19 chicken embryos or hatchling chickens (Table 1). Cardiovascular diseases are a major concern to human health and the complexity of their etiology prevents them from being fully understood and therefore prevented. Congenital heart disease is the most prevalent cause of infant death resulting from birth defects, contributing to more than 24% of total infant mortality due to birth defects (Roger et al. 2011). The establishment of the primary heart tube, looping of the developing heart to the right, and septation to form a four-chamber heart are under the control of multiple signaling pathways. Exogenous agents are capable of disrupting these processes to alter heart development, resulting in malformation. Even if disruption in development does not directly lead to heart malformation, the effects of delayed development or altered function could still contribute significantly to heart diseases later in life. Previous studies report that developmental delays and subsequent catch up in development could increase the incidence of coronary heart disease, obesity, hypertension and type II diabetes (Langley-Evans, 2006 and Eriksson, 2006). PFOA is capable of mimicking endogenous ligands and blocking or activating certain receptors, which are known to include PPARα, retinoid X receptor (RXR), and

other receptors in the steroid hormone receptor superfamily (DeWitt et al. 2009). PPARα is known to be involved in heart development (Steinmetz et al. 2005). Moreover, we cannot eliminate the possibility of other unknown interactions of PFOA with other endogenous ligands. Thus, PFOA is potentially capable of disrupting signaling pathways in heart development and inducing developmental cardiotoxicity by its putative interaction with PPARα.

Cardiac remodeling is an important factor in cardiovascular diseases. Normal ventricle wall thickness is a prerequisite to normal cardiac function. Thickening, thinning, or stiffening of ventricular walls could lead to disturbances in electricity-contraction coupling, induce ischemia and affect hemodynamics, contributing to hypertension (Dukanović et al. 2009). Hypertrophy is observed in many cardiovascular diseases, such as hypertrophic cardiomyopathy (Olivotto et al. 2009), hypertensive heart (Koren et al. 1991) and congenital heart diseases (Buchhorn et al. 2003). All of these diseases are reported to have ventricle dimension and ventricle mass alterations. In our study, similar alterations of cardiac morphology were observed: histopathology demonstrated that the right ventricular wall, especially the layer of dense myosin staining, was affected by developmental exposure to PFOA. Echocardiography showed increased left ventricle dimension and mass, which looked like an early stage hypertensive heart (Koren et al. 1991) even though the relative heart weight remained unchanged; perhaps the thinner wall observed in the right ventricle counterbalanced the increase in the left ventricle mass. Although, it is unknown if these alterations were due to primary alteration of the contractile machinery or secondary to volume/pressure overloading; the similarity between human diseases and our observations in experimental animals suggests the possibility that developmental PFOA exposure might contribute to human cardiovascular diseases, especially congenital heart diseases and/or later cardiovascular diseases including hypertensive heart and cardiomyopathy. However, no

conclusions about the effects of PFOA on cardiac development in humans can be made until a mechanism and its applicability to humans is determined.

Cardiac ultrasound allowed us to directly measure cardiac functional parameters, including heart rate, stroke volume, ejection fraction(fraction of blood pumped out of the right and left ventricles with each heart beat) and fraction shortening (the fraction of any diastolic dimension that is lost in systole). These parameters are important indicators for congenital heart diseases as well as generalized cardiovascular diseases. Neonates with congenital heart disease often have altered heart rate, stroke volume, ejection fraction and fraction of shortening, which are diagnostic tools as well as indicators for treatments (Tsai et al. 2008, Beaufort-Krol et al. 2007 and Lutin et al. 1999). In our study, we found significant alterations of these functional parameters: heart rate was slightly decreased by 0.5 mg/kg of PFOA and increased by over 50% relative to vehicle control in the 1 and 2 mg/kg groups. Stroke volume changes were in the opposite direction: relative to vehicle controls, it was slightly higher at 0.5 mg/kg and lower at the two higher doses. Overall, cardiac output was roughly the same as the vehicle control at all dose groups except for the 1 mg/kg group, which was elevated by 49.9% (data not shown). Meanwhile, ejection fraction and fraction shortening were elevated in all dosed groups, which are consistent with compensatory hypertrophy. Increased left ventricular mass and heart rate are consistent with decreased stroke volume in these animals. Compensation also could possibly explain the thinned right ventricular wall. Increased left ventricular pressure could decrease the blood shunt from pulmonary circulation to main circulation and thus cause volume overload in the right ventricle and a thinning of the right ventricular wall. One factor that we did not measure, but that could have impacted the functional parameters, is a PFOA-induced stress response. As we did not measure serum corticosterone levels in this study, we cannot speculate

about this potential contributor to our results. In future studies, we plan to evaluate cardiac function in adult chickens exposed during development to determine if the dysfunction persists or worsens after heat or exercise-induced stress. However, some toxicological endpoints associated with PFOA exposure, notably immune suppression, do not appear to be stress related (DeWitt et al. 2009). In general, ultrasound data suggest a dose-dependent PFOA-induced ventricular hypertrophy, which is well compensated at one day post-hatch.

Myofibril consists of the whole contractility machinery in the myocardium. Its rate of ATP hydrolysis, i.e., myofibril ATPase activity, is an estimation of cardiac sarcomere function (Lionne et al. 2003) and can be an endpoint of cardiotoxicity (Cappelli et al. 1989). Calcium is an important regulator for myofibril ATPase activity and in disease conditions such as cardiomyopathy, calcium sensitivity can be altered, which ultimately affects myofibril ATPase activity (Chang et al. 2005). In our study, the cardiac myofibril ATPase assay demonstrated that the calcium-dependent ATPase activity did not differ across dose groups, but the calcium-independent ATPase activity increased with the dose of PFOA, thus the ratio of calcium-independent ATPase activity to calcium-dependent ATPase activity increased, (Figure 5). During cardiac hypertrophy, a compensatory mechanism is a reversion to the fetal phenotype and a decrease in myofibril ATPase activity. We observed the opposite, which suggests that in addition to inducing cardiac hypertrophy, developmental PFOA exposure also may affect cardiac energetics. Further investigation is warranted to determine how these events are related.

Our data indicate that developmental exposure to PFOA affects morphology and function of the hearts of hatchling chickens. The similar morphological and functional changes between our avian model and human congenital heart disease as well as generalized cardiovascular diseases suggest that PFOA may have a similar mode or mechanism of action across vertebrate

phylogenetic classes; however, additional work is needed to investigate the mechanism of this endpoint before we can make conclusions about the direct relevancy of our findings to PFOA-exposed humans. However, the observation of left ventricle structure via echocardiography showed structural changes similar to an early stage hypertensive heart; as a result, we cannot eliminate the possibility that PFOA's effect on the heart is secondary to hemodynamic change. Future studies may include assessments of blood pressure. In addition, evaluating adult animals exposed to PFOA during development may provide more detailed information about the developmental basis of adult cardiovascular disease.

As the result of the stewardship program between major fluorochemical manufacturers and the US EPA, PFOA is being phased out by its major U.S. manufacturers. Recent epidemiologic studies have reported that the geometric mean concentration in the general U.S. population has dropped from 4.7 ng/mL in 2000-2001 (Olsen et al. 2003) to 3.44 ng/mL in 2006 (Olsen et al. 2011). However, pockets of high serum concentrations still exist in the U.S. Serum concentrations as high as 88 ng/mL (median of 38.1 ng/mL) have been reported in serum of donated blood from males living in New York state (Kannan et al. 2004). Recently, Hoffman et al. (2011) reported a high concentration of 164.3 ng/mL (median of 75.7 ng/mL) in males from a community surrounding a fluoropolymer production facility (Hoffman et al. 2011). Occupational exposures lead to higher values; serum PFOA concentrations as high as 5,100 ng/mL have been reported (Olsen et al. 2007).

Occupational serum concentrations of PFOA can be four times greater than the serum concentration of the lowest dose group in the present study (1230.8 ng/ml), which suggests that if developing humans are exposed to occupational concentrations of PFOA, a risk of heart anomalies may exist. The serum concentration of PFOA in the low dose group of the present

study is only 14 times greater than the concentration reported for humans with high background concentrations (88 ng/mL) and about 7.5 times greater than the high concentration reported for the population living near a fluoropolymer production facility. This value suggests that developmental cardiotoxicity may be an endpoint of concern for highly exposed (non-occupational) human populations, especially when combined with other risk factors. In a review of epidemiological studies evaluating the health effects of PFOA, Steenland et al. (2010) examined data on cardiovascular disease of workers exposed to PFOA. One study indicated a positive trend between serum PFOA and cardiovascular disease-associated mortality whereas another study indicated no positive trend. Steenland et al. (2010) concluded that data are currently insufficient for inferring associations between PFOA exposure and cardiovascular disease. However, the workers in the studies examined by Steenland et al. (2010) were likely adults during their exposure period. A lack of effects observed in studies of occupationally-exposed adults cannot be taken to mean that developing humans will respond similarly, especially when the increased sensitivity of developing systems relative to adult systems is considered.

As PFOA is present in wild birds, it is also important to consider the implications of our findings on environmental health. The range of PFOA concentrations reported in wild birds is approximately 0.06 to 2 ng/mL (Martin et al. 2004; Bossi et al. 2005 and Falandysz et al. 2007). Generally, levels of PFOA in wild birds are an order of magnitude lower than perfluorooctane sulfonate (PFOS), another PFAA of environmental and human health concern. We observed functional effects in all exposure groups and morphological effects in the two highest dose groups. The average PFOA serum concentration that we observed in our lowest dose group (1230.8 ng/mL) is 2 x 10^4 to approximately 600 times higher than reported concentrations in

wild birds. At current PFOA exposure levels, our study suggests that wild birds are not at risk of developing heart anomalies.

Currently, we are evaluating potential mechanisms behind the effects that we described. PFOA is a known PPARα agonist; agonism of PPARα may regulate expression of proteins involved in fatty acid transport, catabolism, and energy homeostasis (Peters et al. 2005). In addition, as reported by Izumi et al. (2001), bone morphorgenic protein-2 (BMP-2) inhibits apoptosis in neonatal cardiac myocytes. Decreased apoptosis may be a pathway to the hypertrophy that we observed in the hatchling chicken hearts. As PPARα also was reported to interact with the BMP-SMAD pathway (Takano et al. 2011), it is possible that PFOA's developmental cardiotoxicity is initiation through its interaction with PPARα and subsequent alteration of the BMP-SMAD pathway. However, it also is possible that PFOA directly interacts with the BMP-SMAD pathway independent of PPARα. Initial studies with WY-14643, a strong PPARα agonist, indicate that the latter pathway may be more likely than the former. Moreover, because serum/plasma concentrations of PFOS are higher in wild birds than are PFOA concentrations, we plan to repeat the ultrasound studies with chickens exposed to PFOS.

2.5 Conclusions

This study investigated morphological and functional changes in developing chicken hearts following PFOA exposure. A thinner myosin dense layer in the right ventricular wall was observed and a thickened left ventricular wall and increased left ventricular mass was observed by cardiac ultrasound. Alteration in cardiac function also was observed by ultrasound; the decreased stroke volume along with increased heart rate indicates early compensation for cardiac dysfunction. A cardiac myofibril ATPase assay detected an increase in the calcium-independent

ATPase to calcium-dependent ATPase ratio. Our findings suggest that at the doses we administered, PFOA disrupts avian heart development.

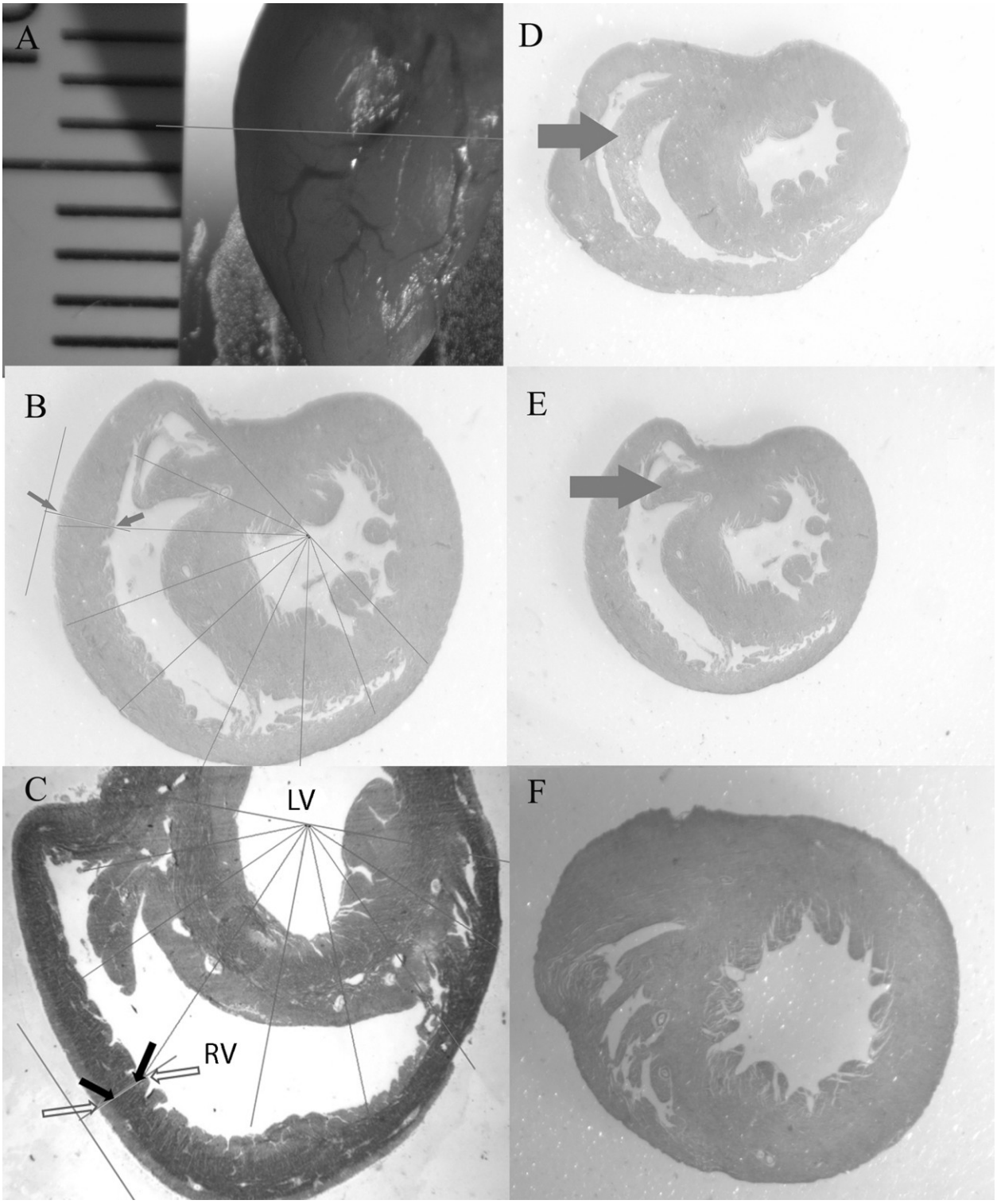

A
D
B
E
C
LV
RV
F

Figure 2.1 Morphological measurement methods.

A: Hearts were cut at approximately 60% of length of heart from ventricular apex. Ruler shows the total length (mm). Transverse line shows position of actual cut.

B: Position of measurements for right ventricular wall thickness (H&E staining). Seven measurements were made on each heart (indicated by radiating lines). Angle between each measurement line was 22.5°. Arrows show measurement points for one of the seven measurements.

C: Position of measurements for whole right ventricular wall and myosin dense layer thickness. RV: right ventricle. LV: left ventricle. Between open arrows: total right ventricular wall thickness. Between closed arrows: myosin dense layer thickness.

D-F: Anatomical marker for relatively constant position on heart sections. D shows a cut at about 70% length of heart from apex. Arrow: marker, which is too long. E shows a cut at 60% length from apex. Arrow: marker, which is size used across all sections. F shows a cut at 50% length from apex; marker absent.

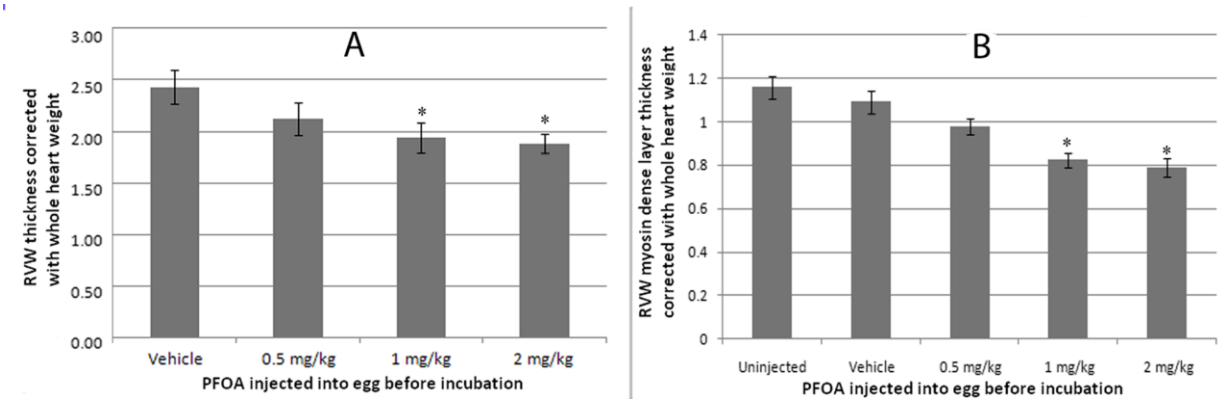
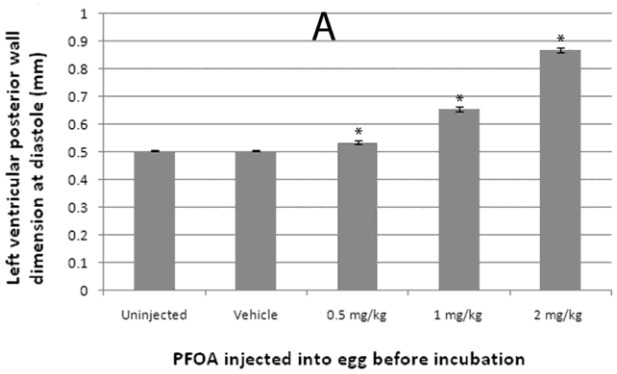


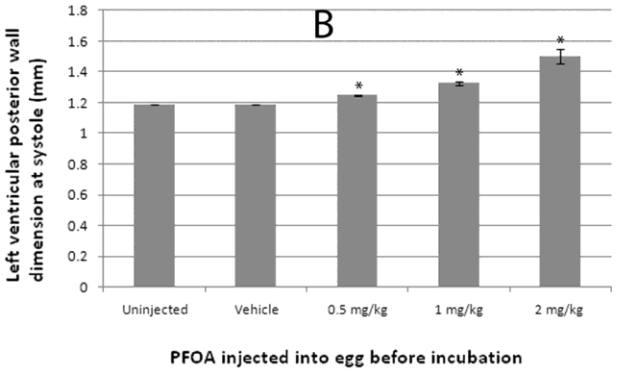
Figure 2.2 Right ventricular wall thickness in hearts of 19-day-old chicken embryos. Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA and incubated to day 19; hearts were sectioned at 6 μm and stained with H&E (A) or for myosin (B).

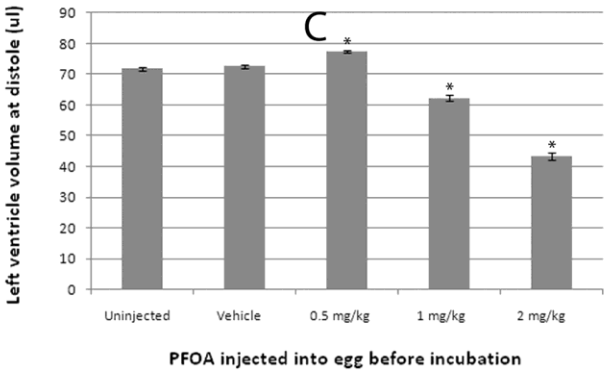
A: Right ventricular wall thickness corrected for whole heart weight (Uninjected group not shown, N=7-10, error bar represents standard error of mean (SEM)).

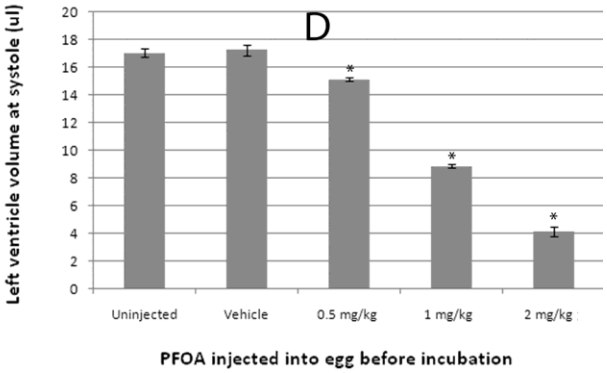
B: Right ventricular wall myosin dense layer thickness corrected with whole heart weight (N=11-15, error bar represents SEM).

*: significantly different from vehicle controls ($P<0.05$).


A
Left ventricular posterior wall dimension at diastole (mm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Uninjected
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
PFOA injected into egg before incubation


B
Left ventricular posterior wall dimension at systole (mm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Uninjected
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
PFOA injected into egg before incubation


C
Left ventricle volume at distole (ul)
90
80
70
60
50
40
30
20
10
0
Uninjected
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
PFOA injected into egg before incubation


D
Left ventricle volume at systole (ul)
20
18
16
14
12
10
8
6
4
2
0
Uninjected
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
PFOA injected into egg before incubation

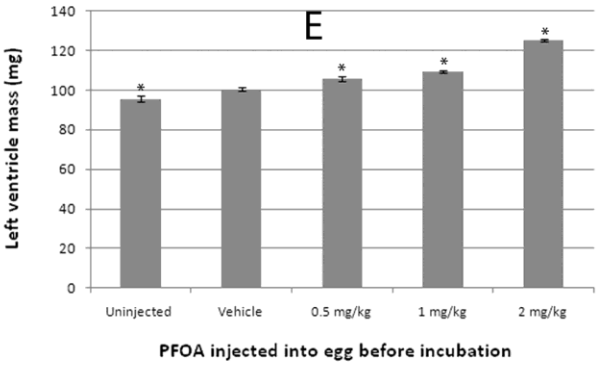

E
Left ventricle mass (mg)
140
120
100
80
60
40
20
0
Uninjected
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
PFOA injected into egg before incubation

Figure 2.3 Cardiac ultrasound of structural parameters in hearts of one-day-old hatchling chickens. (N=9-12, error bar represents SEM). Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA and incubated until hatch. One-day-old hatchling chickens were assessed by echocardiography.

A: Left ventricular posterior wall dimension at diastole.

B: Left ventricular posterior wall dimension at systole.

C: Left ventricle volume at diastole.

D: Left ventricle volume at systole.

E: Left ventricle mass.

*: significantly different from vehicle controls ($P<0.05$).

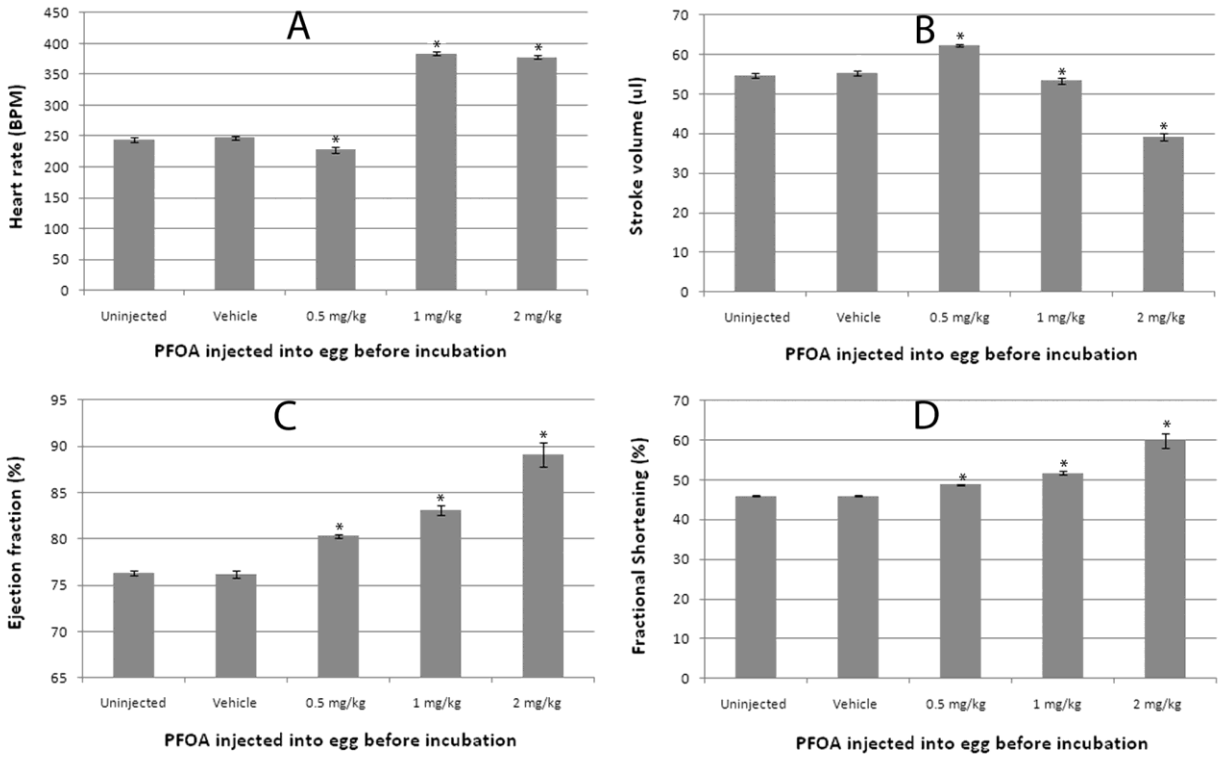


Figure 2.4 Cardiac ultrasound of functional parameters in hearts of one-day-old hatchling chickens. (N=9-12, error bar represents SEM). Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA and incubated until hatch. One-day-old hatchling chickens were assessed by echocardiography.

A: Heart rate.

B: Stroke volume.

C: Ejection fraction.

D: Fraction shortening.

*: Significantly different from vehicle controls ($P<0.05$).

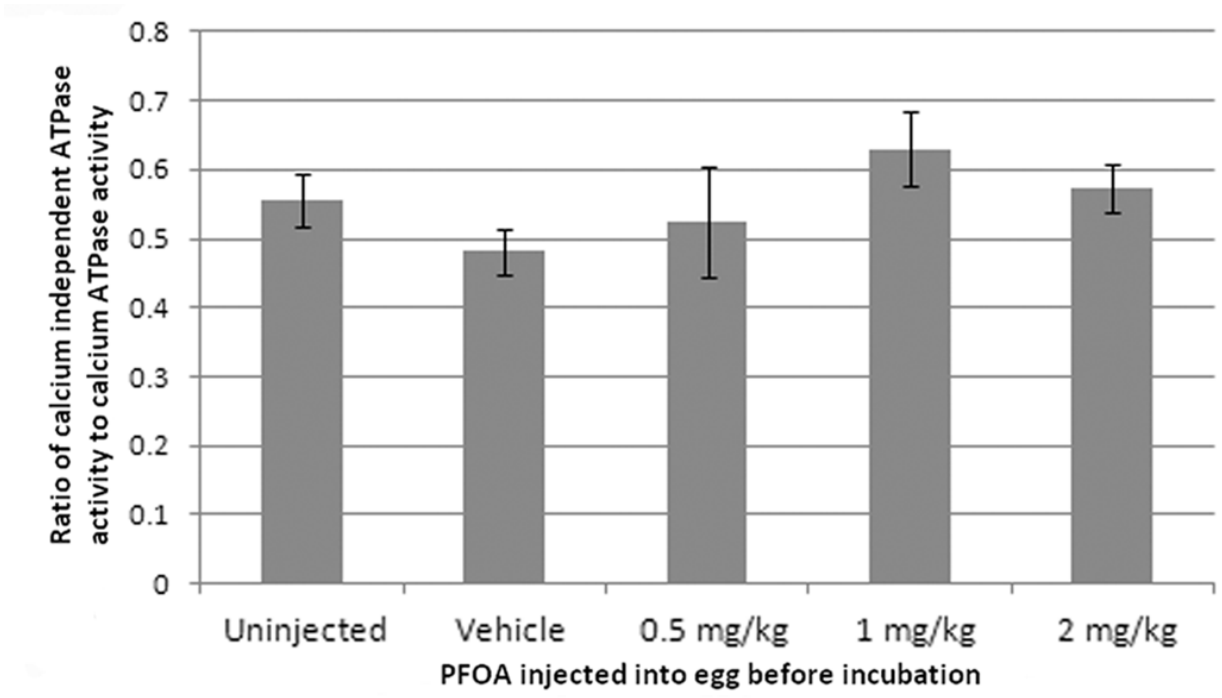


Figure 2.5 Cardiac myofibril ATPase activity in hearts of one-day-old hatchling chickens. Ratio of cardiac myofibril calcium independent ATPase activity to calcium ATPase activity. (N=5 for uninjected group, 7-9 for other groups, error bar represents SEM).

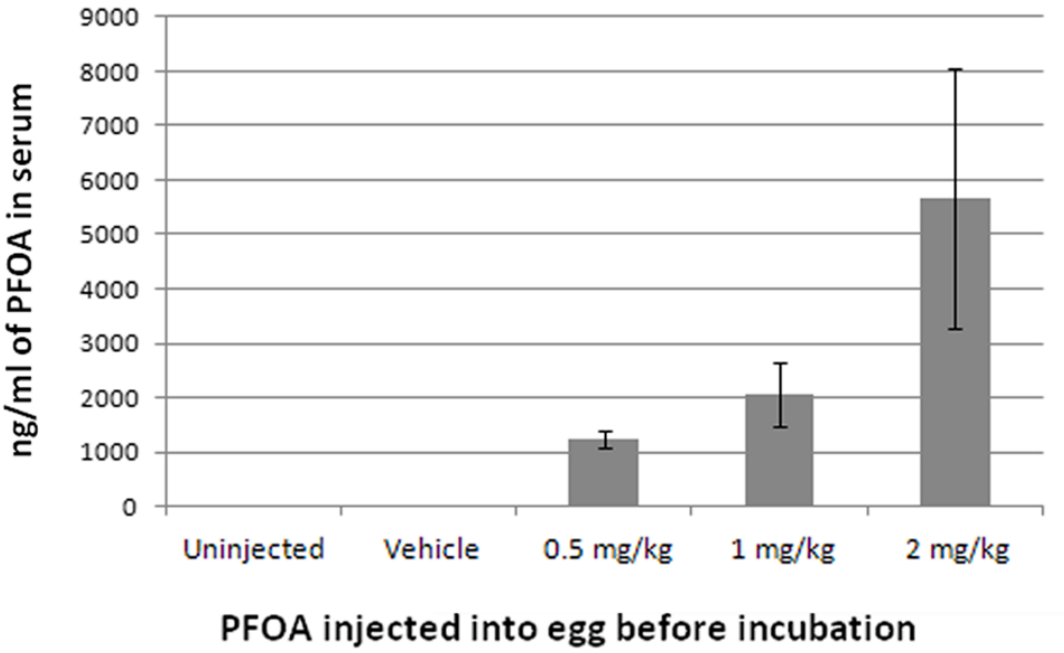


Figure 2.6 Serum PFOA concentrations measured in one-day-old hatchling chickens. Limit of quantitation (LOQ) was 50 ng/mL. All samples from uninjected and vehicle groups were below LOQ and were reported as 0 in the graph (N=2-6, error bar represents SEM).

Table 2.1 Developmental toxicity parameters in 19-day-old chicken embryos and hatchling chickens.

	D19 Embryos[a]				
	Uninjected	Vehicle	0.5 mg/kg	1 mg/kg	2 mg/kg
YFBW[b]	32.30±3.50[c]	30.75±4.15	34.26±4.14	32.74±2.42	32.97±4.47
Relative heart weight[d]	0.0078±0.0014	0.0075±0.00053	0.0078±0.0014	0.0076±0.00037	0.0086±0.0012
Relative liver weight[e]	0.018±0.0030	0.017±0.0052	0.014±0.0044	0.016±0.0030	0.016±0.0022
Mortality	11.4%	26.7%	25.6%	31.4%	46.9%[f]
	Hatchling[g]				
	Uninjected	Vehicle	0.5 mg/kg	1 mg/kg	2 mg/kg
YFBW	36.32±4.45	35.66±2.69	34.51±3.55	35.79±2.50	37.25±4.90
CR length (mm)[h]	8.92±0.59	9.15±1.21	9.38±1.12	9.66±1.15	8.72±0.62
Relative heart weight	0.011±0.0011	0.011±0.0010	0.011±0.0013	0.011±0.00091	0.011±0.0013
Relative liver weight	0.024±0.0039	0.021±0.0052	0.027±0.0032[f]	0.027±0.0050[f]	0.025±0.0028
Mortality[i]	22.2%	33.3%	45.5%	39.1%	37.5%
Hatchability	83.3%	64.3%	64.3%	66.7%	58.3%

[a] N = 6 to 9 per dose group.
[b] Yolk-free body weight.
[c] Mean ± standard derivation.
[d] Relative heart weight = heart weight/YFBW.
[e] Relative liver weight = liver weight/YFBW.
[f] Statistically different from vehicle control grouip ($P < 0.05$).
[g] N = 26 for uninjected group and 9 to 12 for other groups.

[h] Crown to rump length.
[i] Note that mortality increased between D19 and hatching in the 0.5 mg/kg and 1 mg/kg groups.

CHAPTER 3 PERFLUROOCTANOIC ACID INDUCED DEVELOPMENTAL CARDIOTOXICITY: ARE PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR α (PPARα) AND BOND MORPHORGENIC PROTEIN 2 (BMP2) PATHWAYS INVOLVED?

Abstract

Perfluorooctanoic acid (PFOA) is an environmental contaminant known to induce developmental toxicity in animal models through activation of the peroxisome proliferator activated receptor α (PPARα). Previously, it was demonstrated that in ovo exposure to PFOA induced cardiotoxicity in chicken embryos and hatchlings. To investigate potential PPARα-mediated mechanisms, fertile chicken eggs were injected prior to incubation with WY 14,643, a PPARα agonist. Cardiac morphology and function were evaluated in late stage embryos and hatchlings. Histologically, unlike PFOA, WY 14,643 did not induce thinning of the right ventricular wall. Via echocardiography, however, WY 14,643 induced effects similar to PFOA, including increased left ventricular wall thickness and mass, elevated heart rate, ejection fraction, fractional shortening, and decreased stroke volume. Additionally, to investigate mechanisms associated with early heart development, a separate group of fertile chicken eggs was injected prior to incubation with PFOA or WY 14,643 and in early stage embryos, gene expression and protein concentration associated with the bone morphogenic protein (BMP2) pathway was determined. Although changes were not statistically consistent among doses, expression of BMP2, Nkx2.5, and GATA4 mRNA in early embryos was altered by PFOA exposure; however, protein concentrations of these targets were not markedly altered by either PFOA or WY 14,643. Protein levels of pSMAD1/5, a transcriptional regulator stimulated by BMPs, were altered by both PFOA and WY 14,643, but in different directions; PFOA reduced cytoplasmic pSMAD1/5

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whereas WY 14,643 decreased nuclear pSMAD1/5. Taken together, these data suggest that developmental cardiotoxicity induced by PFOA likely involves both PPARα and BMP2 pathways.

3.1 Introduction

Perfluorooctanoic acid (PFOA) is a fluorinated compound that is used as a polymerization aid in the manufacture of many fluorinated polymers and elastomers, which are then used in myriad consumer and industrial products. PFOA is persistent in environmental media (Vaalgamaa et al. 2011) and is detectable in biota as well as serum of the general human population. The median human serum concentration in the U.S. general population was 4.3 ng/ml in 2007-2008 (CDC 2012). However, in areas with contaminated drinking water, serum concentrations are higher. Shin et al. (2011) reported a median serum value of 24.3 ng/ml in residents living in an area of high contamination in West Virginia and Ohio. Another study reported a median serum value of 75.7 ng/ml in residents of communities surrounding a fluoropolymer production facility (Hoffman et al. 2011). Serum concentrations as high as 5100 ng/ml have been reported in occupationally-exposed humans (Olsen et al. 2007). Because of the persistence of PFOA and related compounds in environmental and human samples, the U.S. Environmental Protection Agency (USEPA) worked with 8 major manufacturers of PFOA to establish a stewardship program to eliminate PFOA and related compounds from emissions and products by 2015 (USEPA 2012). However, exposure likely will continue for a considerable time due to the environmental persistence and long half life of PFOA in humans.

Epidemiological studies of the effects of PFOA on human development have been conducted in areas of known high exposures such as West Virginia-Ohio (U.S.) (Frisbee et al.

2009) and the Faroe Islands (Denmark) (Grandjean et al. 2012). Modest associations of PFOA serum concentrations with preeclampsia and birth defects were found in the Mid-Ohio Valley population (Stein et al. 2009). PFOA exposure was also associated with elevated serum cholesterol and uric acid levels in children and adolescents from this population (Frisbee et al. 2010). However, Nolan et al. (2009; 2010) reported that elevated PFOA exposure in this population was not associated with lowered birth weight or gestational age, labor or delivery complications, or maternal risk factors such as eclampsia or diabetes, although lack of umbilical cord serum PFOA concentrations made it difficult to assess actual fetal exposures. In a recent study, developmental exposure to a mixture of perfluorinated compounds (PFCs) was associated with decreased responses to vaccinations in children between 5 and 7 years of age living in the Faroe Islands (Grandjean et al. 2012). In laboratory animal models, PFOA is known to induce hepatic, pancreatic, and testicular cell cancers (Biegel et al. 2001), endocrine disruption (Olsen et al. 1998), and immunotoxicity (DeWitt et al. 2008). In addition, PFOA was reported to induce developmental effects, including retarded development and decreased fetal survival (Wolf et al. 2007). The mechanisms underlying retarded development and decreased fetal survival remain to be elucidated. It was postulated that as the cardiovascular system is one of the earliest organ systems to develop, cardiotoxicity induced by developmental PFOA exposure may contribute to decreased fetal survival.

Previously, Jiang et al. (2012) observed developmental cardiotoxicity in an avian model. Following in ovo developmental exposure to PFOA, hearts from near-hatch (embryonic day 19; ED19) domestic chicken embryos exhibited morphological and functional changes, including thinning of the right ventricular wall and of a layer of dense myosin staining within the right

ventricular wall, increased left ventricle dimension, and altered left ventricular function. The underlying mechanism(s) of this developmental cardiotoxicity remain unknown.

It is generally accepted that PFOA activates PPARα (Rosen et al. 2008). PPARα is a ligand-activated transcription factor in the steroid hormone superfamily that regulates fatty acid metabolism, a process that may induce several effects on cardiac tissue (Djouadi et al. 1999). To our knowledge, PPARα activation is not a major pathway or transcription factor involved specifically in heart development nor is its activation known to directly interfere with the major pathways associated with heart development. Regardless, evidence exists that PPARα activation has the potential to affect the cardiovascular system. Yuan et al. (2008) reported that PPARα activation attenuated myocardial damage induced by isoproterenol, a medication used to treat bradycardia, in adult Wistar rats. Another study indicated that mutation of the PPARα gene resulted in left ventricular diastolic dysfunction (Juang et al. 2010). It is possible that PPARα activation plays a role in the developmental cardiotoxicity induced by PFOA. However, not all toxicities induced by PFOA exposure require PPARα; PFOA-induced suppression of antigen-specific antibody responses occured at similar doses in both wild-type and constitutive PPARα knock-out C57BL/6 mice (DeWitt et al. 2008). Therefore, the aim of this study was to explore the potential mechanisms underlying of PFOA-induced developmental cardiotoxicity in an avian model by examining pathways associated with PPARα agonism, pathways known to be associated with PFOA exposure, and pathways known to be critical for early heart development.

3.2 Methods and materials

3.2.1 Overall methods

3.2.1.1 Chemicals

Sunflower oil was purchased from Spectrum Organic Products, LLC (Boulder, CO). PFOA, WY 14, 643, and other chemicals (if not otherwise mentioned) were purchased from Sigma–Aldrich (St. Louis, MO).

3.2.1.2 Animals

Fertile chicken (*Gallus gallus*) eggs were purchased from the North Carolina State University Poultry Research Center (Raleigh, NC). Eggs were cleaned with 20% povidone iodine, candled to outline air cells in pencil on shells, weighed, given ID numbers, and evenly distributed by weight among doses. Uninjected eggs were included in each batch as environmental controls.

3.2.1.3 Egg injection and incubation

Egg injection and incubation were carried out as described previously (Jiang et al. 2012). Briefly, PFOA or WY 14,643 was suspended in sunflower oil and vortexed before injection into the air cell of each egg. Ten percent ethanol was added to the WY 14,643 solutions to improve its solubility in oil. Stock solutions were prepared so that 0.1 µl of solution per g egg weight resulted in the appropriate mg/kg egg weight concentration. After injection, eggs were incubated in a Lyon Roll-X incubator (Chula Vista, CA) set at 37.5–37.8 ºC dry bulb and 30.6–31.1 ºC wet bulb.

3.2.1.4 Sample collection for late stage embryos and hatchlings

Eggs incubated until ED19 or hatch were candled every 2-3 days so that infertile/undeveloped/dead eggs could be removed. After external pipping, eggs were placed

individually into containers large enough for the hatched chickens and transferred to a larger incubator (G.Q.F. Manufacturing Co., Savannah, GA). Hatchling chickens were kept in a warmed brood box until euthanasia; all echocardiography measurements were collected on hatchlings within 24 hr of hatch. On ED19, chicken embryos were removed from eggs and quickly decapitated. Hearts were collected, rinsed in cold saline, weighed, and fixed in formaldehyde for histological analysis. Livers were collected, weighed, frozen on dry ice, and then stored at -80ºC for later use. Hatchling chickens were anesthetized with carbon dioxide and quickly decapitated. Serum, heart, and liver were collected and stored at -80ºC for later use. All procedures were approved by the East Carolina University Institutional Animal Care and Use Committee.

3.2.1.5 Sample collection for early stage embryos

Two groups of early stage embryos were collected; one group included whole embryos collected on ED4 and another group included isolated hearts from ED6 embryos. On ED4, whole embryos were removed from eggs, dissected to remove the amnionic membrane, snap frozen in liquid nitrogen, and then stored at -80ºC for later use. On ED6, whole embryos were removed from eggs; hearts were dissected out, snap frozen in liquid nitrogen, and then stored at -80ºC for later use.

3.2.1.6 Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed with the SAS JMP System (SAS Institute, Cary, NC). Endpoints were analyzed by one-way analysis of variance (ANOVA) by dose and when ANOVA results indicated statistically significant dose effects, individual post hoc comparisons were made with a two-tailed t-test. Statistical significance was determined when P-values were less than 0.05. For the western blot

sample collection, samples were collected in two batches for whole embryos collected at ED4 and another two batches for hearts collected from ED6 embryos due to size limitations of the egg incubator. These batches were statistically compared before pooling, which verified that the results of each batch did not differ statistically.

3.2.2 Methods specific to PPARα agonism by WY 14,643

The following section describes experiments done only with eggs injected with WY 14,643 to investigate the role of PPARα agonism in developmental cardiotoxicity.

3.2.2.1 Echocardiography on hatchling chickens

Echocardiography was used to assess cardiac morphology and function in hatchling chickens exposed to WY 14,643. Briefly, hatchling chickens were evaluated with Vevo 2100 (Visualsonics, Toronto, Ontario, Canada) within 24 hours post-hatch. Hatchlings were enclosed in stockinettes for proper restraint without feather damage. A hole was cut on the stockinette to expose the chest so that echocardiography could be carried out. For detailed procedures please refer to Jiang et al. (2012).

3.2.2.2 Histology

Histology on hearts from ED19 chickens treated with vehicle or WY 14,643 was carried out as previously described (Jiang et al. 2012). Briefly, ventricles were paraffin embedded and sliced into 6 μ sections on a rotary microtome. One subset of the sections was stained with hematoxylin and eosin for morphological measurement of the thickness of the right ventricular wall. Another subset of the sections was stained immunohistochemically with myosin for measurement of the thickness of a dense layer of myosin staining present in the right ventricular wall. Figure 3.1A illustrates the right ventricular wall thickness and the thickness of a dense layer of myosin staining.

3.2.2.3 Quantitative real-time PCR

Livers from hatchling chickens were assessed with quantitative real-time PCR for expression of liver fatty-acid-binding protein (L-FABP), a transcript indicative of PPARα activation that can be used to confirm exposure of WY 14,643 to embryos (Karam and Ghanayem, 1997). While the CYP4A family genes are more commonly used as PPARα activation confirmation in rodents, avian species do not express CYP4A. RNeasy Mini Kits, QuantiTect Reverse-Transcription Kit, SYBR green, and primers were purchased from Qiagen (Valencia, CA). RNAspin Mini Kits were purchased from GE Healthcare (Little Chalfont, UK) and Total RNA mini kits were purchased from IBI scientific (Peosta, IA). Additionally, SYBR green mastermix and iScript Advanced cDNA Synthesis Kits were purchased from Bio-Rad Laboratories (Hercules, CA). RNA isolation was carried out according to manufacturer's protocols. A Nanodrop ND-1000 (Wilmington, DE) was used to determine the concentration and quality of mRNA. For reverse transcription, manufacturer's protocols were followed and 1 µg mRNA used for each sample. Quantitative real-time PCR was carried out in 25 µl (Qiagen SYBR Green) or 20 µl (Bio-Rad SYBR Green). The PCR reactions were performed in an Applied Biosystems StepOne Plus (Foster City, CA) or Bio-Rad CFX96 Real-Time PCR machine (Hercules, CA). Results were analyzed with the two delta-delta-CT method, normalizing to GAPDH for fold changes relative to the vehicle control group.

3.2.2.4 Western blot

Early stage embryos exposed to WY 14,643 were analyzed with western blot to measure levels of pSMAD1/5 (in ED4 whole embryos), Nkx2.5, and pGATA4 proteins (in ED6 hearts). Cytoplasmic and nuclear proteins from whole ED4 embryos were separated with an NXTRACT kit, with the addition of 25mM beta-glycerophosphate. Hearts isolated from ED6 embryos were

homogenized in RIPA buffer (sodium chloride 150 mM, Tris-HCl 50 mM, SDS 0.1%, sodium deoxycholate 0.5% and triton X-100 1%) with 0.5 mM dithiothreitol (DTT), 25mM beta-glycerophosphate, and 1:100 protease inhibitor cocktail (Thermo Scientific, Rockford, IL), extracted in a cold room (4°C) for 2 hr and then centrifuged at 15000xg for 10 min. Primary antibodies included anti pSMAD1/5 (Invitrogen, Grand Island, NY), anti nkx2.5 (Abcam, Cambridge, MA), and anti pGATA4 (Invitrogen, Grand Island, NY), all applied at 1:1000; anti-beta actin was applied at 1:10000. Secondary antibodies (LI-COR Biosciences, Lincoln, NE) were all applied at 1:15000. Visualization and semi-quantification was conducted with a LI-COR Odyssey system (LI-COR Biosciences, Lincoln, NE), all target proteins were normalized to beta actin and compared to vehicle control for relative fold change.

3.2.3 Methods specific to PFOA-exposed embryos

The following section describes experiments conducted with eggs only injected with PFOA to investigate the role of pathways associated not only with potential PPARα agonism by PFOA, but pathways associated with PFOA exposure and early heart development. These pathways included inflammatory cytokines and BMP2-pSMAD1/5-Nkx2.5/GATA4 pathways.

3.2.3.1 Quantitative real-time PCR

Early stage embryo samples (ED4 whole embryos and hearts isolated from ED6 embryos) were assessed with quantitative real-time PCR for expression of various genes following PFOA exposure. Reagents and methods used were the same as described in the previous section. Targets included GAPDH, PPARα, BMPR1A, LITAF, IL1-β, IL-6, BMP2, RXRα, Nkx2.5 and GATA4.

3.2.3.2 Western blot

Proteins isolated from early stage embryo samples exposed to PFOA were analyzed with western blot to measure levels of pSMAD1/5 (in ED4 whole embryos), Nkx2.5, and pGATA4 proteins (in ED6 hearts). Reagents and methods were the same as described in the previous section.

3.3 Results

3.3.1 PPARα agonism by WY 14,643

3.3.1.1 General toxicity

Mortality, hatchability, yolk-free body weight, absolute heart weight, and relative heart and liver weights for both ED19 embryos and hatchling chickens following exposure to WY 14,643 are reported in Table 3.1. In ED19 embryos, no significant changes were observed among the parameters measured. In hatchling chickens exposed to 25 mg of WY 14,643/kg egg weight, relative heart weight was significantly elevated by 18.3% relative to the vehicle group. No other differences in general toxicity parameters were observed.

3.3.1.2 Histology

In ovo exposure to WY 14,643 did not induce a thinning of the right ventricular wall or of a dense layer of myosin staining in the right ventricular wall of ED19 chicken embryos. Representative histology sections are shown in Figures 3.1B, C and D. As illustrated in Figures 3.1E and 1F, no marked differences among dose groups were detected.

3.3.1.3 Echocardiography

Echocardiography revealed morphological and functional alterations in hearts of hatchling chickens exposed to WY 14,643 during development, relative to hearts of vehicle-

exposed hatchlings. As shown in Table 3.2, exposure to WY 14,643 increased left ventricular posterior wall dimension at diastole by 21.4% (5 mg/kg egg weight) and 96.7% (25 mg/kg egg weight). At systole, exposure to WY 14,643 elevated left ventricular posterior wall dimension by 20.8% (5 mg/kg egg weight) and 44% (25 mg/kg egg weight). The volume of the left ventricular volume was reduced by 13.8% (5 mg/kg egg weight) and 36.4% (25 mg/kg egg weight) at diastole. At systole, left ventricular volume was decreased by 45.8% (5 mg/kg egg weight) and 72.3% (25 mg/kg egg weight). In addition, left ventricle mass was increased by 23.9% (5 mg/kg egg weight) and 44.4% (25 mg/kg egg weight).

Exposure to WY 14,643 also affected how the hearts of the hatchling chickens functioned. Heart rate was elevated by 48.1% (5 mg/kg egg weight) and 59.8% (25 mg/kg egg weight) and stroke volume was reduced in the 25 mg/kg egg weight group (26.1%). Cardiac output was elevated by 63.2% (5 mg/kg egg weight) and 85.0% (25 mg/kg egg weight). The ejection fraction was elevated by 10.3% (5 mg/kg egg weight) and 15.8% (25 mg/kg egg weight). Fractional shortening was increased by 6.2% (25 mg/kg egg weight).

3.3.1.4 Quantitative real-time PCR

In livers of hatchling chickens exposed to WY 14,643, L-FABP mRNA was significantly increased by 87.1% following exposure to 25 mg/kg egg weight (Figure 3.2) but not by 5 mg/kg egg weight. Further, in a subset of livers collected from hatchling chickens exposed to 2 mg/kg egg weight of PFOA, L-FABP was not markedly elevated.

3.3.1.5 Western blot

In protein extracted from ED4 whole embryos exposed to WY 14,643, the nuclear pSMAD1/5 level was significantly decreased by 22.2%, on average (Figure 3.3D). No marked difference was detected in cytoplasmic levels of pSMAD1/5 (Figure 3.3C). No significant

differences were detected in levels of Nkx2.5 (Figure 3.5A) or pGATA4 (Figure 3.5B) proteins when hearts of ED6 embryos were examined.

3.3.2 PFOA-exposed embryos

3.3.2.1 Quantitative real-time PCR

Exposure to PFOA did not markedly alter expression of PPARα, BMPR1A, LITAF, IL1-β, IL-6 and RXRα mRNA in ED4 whole embryos (data not shown). BMP2 (Figure 3.4A) was significantly decreased by 31.9% in embryos treated with 0.5 mg/kg egg weight relative to vehicle control, but not in any other dose group or in hearts collected from ED6 embryos. Following PFOA exposure, expression of Nkx2.5 (Figure 3.4B) and GATA4 (Figure 3.4C) mRNA was significantly elevated in hearts collected from ED6 embryos. Nkx2.5 was increased by 60.7% after exposure to 2 mg/kg egg weight; GATA4 was elevated by 78%, on average, after exposure to all doses of PFOA.

3.3.2.2 Western blot

In protein extracted from ED4 whole embryos exposed to PFOA, cytoplasmic levels of pSMAD1/5 were significantly decreased by an average of 32.5% after exposure to 1 or 2 mg/kg egg weight (Figure 3.3A). No marked difference was detected in nuclear levels of pSMAD1/5 (Figure 3.3B). No marked differences were detected in levels of Nkx2.5 (Figure 3.5A) or pGATA4 (Figure 3.5B) proteins when hearts of ED6 embryos were examined.

3.4 Discussion

3.4.1 Dose selection

Dose selection for PFOA was based on observations by O'Brien et al. (2009) that up to 10 mg of a PFOA injection/kg of egg weight did not affect pipping success and on previous

experiments done in our lab. From our previous study (Jiang et al. 2012), air cell injection of 2 mg of PFOA/kg of egg weight prior to incubation resulted in serum concentrations of 5670 ng/ml in hatchling chickens, which is higher levels in the general human population but comparable to levels in occupationally- exposed humans. Dose selection for WY 14,643 was based on a study by Woods et al. (2007) and on pilot studies in our lab. Woods et al. (2007) gave WY 14,643 by oral gavage to C57B6/J and SV129 mice at 5 mg/kg and 50 mg/kg without acute complications, and observed significant increases in downstream genes regulated by PPARα activation, including Acyl-CoA oxidase. In the current study, the doses applied 0, 5 or 25 mg of WY 14,643/kg egg weight did not increase mortality and 25 mg/kg was the highest achievable dose for this procedure, based on the solubility of WY 14,643 in oil. Note that as WY 14,643 is metabolized during development, it is impossible to obtain accurate serum concentrations in hatchling chickens for comparisons to PFOA serum concentrations.

3.4.2 Comparison between WY 14,643 and PFOA on PPARα activation

WY 14,643 is a PPARα agonist; as low as 0.3 mg/kg will fully activate PPARα in the mouse, but about 18 mg/kg is needed to fully activate PPARα in humans (Maloney and Waxman, 1999). The high dose of WY 14,643 applied (25 mg/kg) was designed to activate PPARα to the maximum extent without inducing marked embryonic mortality. mRNA expression of L-FABP confirmed that PPARα was activated sufficiently following exposure to 25 mg/kg of WY 14,643. PFOA has a lower affinity for PPARα. And at least 2-4 mg/kg is needed to fully activate the mouse PPARα (Maloney and Waxman, 1999). No published data have elaborated the ability of WY 14,643 or PFOA to activate the avian isoform of PPARα. From a previous pilot study in our lab, Acyl CoA oxidase activity did not increase in livers isolated from chicken embryos of various ages exposed to 0, 0.5, 1, or 2 mg of PFOA/kg of egg

weight prior to the start of incubation (data not shown). These pilot data suggest limited PPARα activation in chickens. However, 2 mg/kg of egg weight was the highest PFOA dose in this procedure that did not significantly induce mortality and general toxicity. This dose of PFOA did increase mean L-FABP mRNA expression (although not markedly), indicating mild PPARα activation by PFOA. However, as doses of PFOA greater than 2 mg/kg induce embryonic mortality and general toxicity, as demonstrated by our previous studies (Jiang et al. 2012), it is likely that PPARα activation by PFOA is not a major mechanism of embryonic mortality in a chicken model.

3.4.3 PPARα agonism and developmental cardiotoxicity

The role of PPARα agonism in developmental cardiotoxicity was investigated by using the PPARα agonist WY 14,643. Numerous studies have reported effects of WY 14,643 exposure on the heart, including inhibition of mitochondrial respiration in isolated rat cardiac mitochondria (Zungu et al. 2006), altered fatty acid composition of myocardial lipids (Baranowski et al. 2009), and attenuation of cardiomyocytic apoptosis following cardiac ischemia and reperfusion (Yeh et al. 2006). Developmental exposure to WY 14,643 did not increase the mortality rate in ED19 chicken embryos or hatchling chickens. Developmental exposure to PFOA, however, elevated the mortality rate in ED19 chicken embryos at the highest dose administered (2 mg of PFOA/kg of egg weight), but not in hatchling chickens (Jiang et al. 2012). The difference in mortality between PFOA and WY 14,643 in chicken embryos and hatchlings suggests that developmental toxicity of PFOA, in an avian model, is not fully dependent on PPARα agonism. In mammalian models, certain developmental endpoints affected by PFOA exposure require expression of PPARα (Abbott et al. 2007). However, not all measures of PFOA-induced developmental toxicity in mammalian models may be dependent on PPARα

agonism as activation of PPARα by WY 14,643 did not affect developmental toxicity in a mouse model (Palkar et al. 2010). Changes in expression of PPARα in early stage embryos following PFOA exposure were not noted.

Developmental exposure to WY 14,643 did not induce detectable histological thinning of the right ventricular wall (Figure 1) in hearts from ED19 chicken embryos, while developmental PFOA exposure did (Jiang et al. 2012). However, echocardiography demonstrated that developmental exposure to WY 14,643 induced effects similar to PFOA (Jiang et al. 2012). One such effect was an enlarged left ventricle and higher-than-normal functional parameters, which are indicative of morphological and functional alterations observed in a compensating hypertensive heart. Previously, it was postulated that the thinned right ventricular wall resulted from enlargement of the left ventricle, possibly from elevated circulation pressure. This also may be the case after exposure to WY 14,643, although the effects were not sufficiently pronounced to be detectable histologically, suggesting that agonism of PPARα may play an important, but not singular, role in developmental cardiotoxicity observed in chicken embryos and hatchlings exposed to PFOA. mRNA levels of L-FABP were higher in WY 14,643 exposed than in PFOA exposed embryos (Figure 2), indicating that WY 14,643 more potently activates PPARα in avians than does PFOA at the doses applied. Therefore, other pathways and/or processes, in addition to PPARα activation, likely contribute to cardiac changes noted in PFOA-exposed avian embryos.

3.4.4 Other pathways to PFOA-induced developmental cardiotoxicity

While the experiments with WY 14,643 suggest a role for PPARα agonism in developmental cardiotoxicity, exposure to WY 14,643 did not completely mimic the effects of PFOA. Therefore, additional pathways, including those independent of PPARα, likely exist.

3.4.4.1 Inflammatory cytokines

While PPARα activation is not among the classical pathways involved in heart development (Kirby 2002), such as activin/TGF-beta and BMP-2/BMP-4 (Ladd et al. 1998), FGF family (Zhu et al. 1996), Nkx2.5 (Schultheiss et al. 1995), GATA4 (Charron and Nemer 1999) and MEF-2 (Black and Olson 1998), PFOA affects expression of inflammatory cytokines (DeWitt et al. 2009). However, there are contradictory reports regarding the effects of PFOA on inflammatory cytokines. While Taylor et al. (2005) reported that PFOA has potency similar to dexamethasone, a known anti-inflammatory agent, against carageenan-induced edema and thermal hypersensitivity, Yang (2010) found that PFOA exposure increased expression of various inflammatory cytokines in Japanese medaka. The induction/suppression of inflammatory cytokine production may potentially contribute to the developmental cardiotoxicity observed with PFOA. Inflammatory cytokines might interfere with signaling pathways that are important to heart development. IL-1β was found to be able to modulate TGF-beta signaling via IL-6 activation (Luo et al. 2009) and increased TNF-alpha could suppress the TGF-beta receptor population (Yamane et al. 2003). TGF-beta superfamily signaling pathways are crucial for heart development; induction in inflammatory cytokines might contribute to development cardiotoxicity induced by PFOA. While a difference in mRNA expression was not detected for the inflammatory cytokines in early stage embryos, one cannot discount disruption of inflammatory pathways as a contributory factor in PFOA-induced developmental cardiotoxicity. Only limited information is available for early chicken embryo cytokine expression profile. Meade et al. (2009) reported IL-1β expression decreased from ED3 to ED9, then rose to comparable levels to ED3 after ED10. The rapid change of IL-1β and possibly expression of

other cytokines in early chicken embryo development may have prevented detection of changes induced by PFOA exposure.

3.4.4.2 Bone morphogenetic protein 2

BMP2 mRNA was decreased at the lowest administered dose of PFOA in whole embryos collected at ED4, indicating that BMP2 may be involved in PFOA-induced developmental cardiotoxicity. This alteration was not observed in hearts isolated from ED6 embryos, suggesting that interference with BMP2 may occur outside of the heart proper and that the expression of BMP2 is likely dynamic, and possibly transient, similar to observations in the mouse embryo cranial base, where BMP2 mRNA expression area and levels constantly changed from ED10 to postnatal day 5 (Kettunen et al. 2006). Although alterations of ligand expression by itself are not sufficient to conclude that a particular signaling pathway contributes to a specific endpoint, further evidence from the western blot for pSMAD1/5, the activated form of second messengers in the BMP2 pathway, suggests that this pathway may be affected by PFOA exposure. SMAD are second messengers that normally exist in the cytoplasm. On activation, they are phosphorylated, form polymers, and enter the nucleus to as act as transcription factors. By separating the cytoplasmic from the nuclear proteins, additional information about second messenger activation and nuclear trafficking was revealed. Interestingly, PFOA and WY 14,643 induced differential responses for pSMAD1/5. WY 14,643 is known to be a full PPARα agonist, the decreased nuclear pSMAD1/5 concentration suggests that PPARα activation affects the BMP2-SMAD1/5 pathway and potentially decreased the amount of available heteromeric SMAD complex in the nucleus. However, PFOA did not affect the nuclear pSMAD1/5; instead, it decreased the amount of cytoplasmic pSMAD1/5, potentially decreasing the available second messenger pool. The differential effects could be due to different affinity for PPARα and thus a

different extent of PPARα activation or that PPARα-independent mechanisms also contribute to the effect induced by PFOA. These data therefore provide evidence that the BMP2-SMAD1/5 pathway is involved in PFOA-induced developmental cardiotoxicity because altered BMP2 expression and second messenger levels following PFOA exposure were observed. The precise time where PFOA interferes with this signaling pathway remains unknown.

The BMP2 signaling pathway induces both Nkx2.5 and GATA4 expression in developing embryos (Barron et al. 2000). Nkx2.5 is a transcription factor that plays important roles in the regulation of cardiac development, including early specification and proliferation of cardiac precursors and later events such as the formation of the outflow tract (McCulley et al. 2012; Harvey et al. 2002). GATA4 is another transcription factor that is crucial in cardiac morphogenesis and cardiomyocyte proliferation. Decreased GATA4 expression could lead to common atrioventricular canal (CAVC), double outlet right ventricle (DORV), and hypoplastic ventricular myocardium (Pu et al. 2004). Loss of Nkx2.5 and GATA4 lead to heart malformation and lethality (Tanaka et al. 1999; Kuo et al. 1997). In the current study, elevated expression of Nkx2.5 and GATA4 mRNA, but not protein levels, was observed in hearts isolated from ED6 embryos exposed to PFOA during this early stage of development. This is additional evidence that the BMP2-SMAD pathway may be involved in developmental cardiotoxicity induced by PFOA. Nkx2.5 and GATA4 exert hypertrophic effects when upregulated (Li et al. 2011). Higher mRNA expression of Nkx2.5 and GATA4 may partially contribute to the left ventricular hypertrophy and/or the right ventricular wall thinning noted. However PFOA exposure did not markedly alter Nkx2.5 or phosphorylated GATA4 (S105) (pGATA4, the activated form of GATA4) protein levels. Perhaps potential differential expression levels of protein and mRNA or fast degradation/turnover of protein prevented detection of the difference. One possible

explanation for the lack of difference in GATA4 protein concentration may be that phosphorylated GATA4 remains the same whereas the GATA4 pool is changed. However, this remains untested based on antibody availability for an avian model.

Our findings indicate that the BMP2-SMAD1/5-Nkx2.5 and GATA4 signaling pathways are altered by developmental PFOA exposure. These pathways therefore may be involved in PFOA-induced developmental cardiotoxicity in an avian model. However, additional studies are required to further elucidate the precise role of PPARα, BMP2 and Nkx2.5/GATA4 in this developmental cardiotoxicity.

3.4.4.3 Interaction between PPARα and other pathways

The current study illustrated that multiple pathways may contribute to PFOA-induced developmental cardiotoxicity. Data from WY 14,643 experiments indicated that PPARα is one of the pathways that likely contributes to the cardiotoxicity, but because WY 14,643 did not fully mimic the effects of PFOA, other pathways likely exist. Furthermore, alterations in the BMP2 ligand, second messenger, and downstream genes were observed following PFOA exposure, suggesting that BMP2-SMAD-Nkx2.5/GATA4 also may contribute to PFOA-induced developmental cardiotoxicity. Moreover, the PPARα and BMP2 pathways seemed interconnected, as indicated by the effect of WY 14,643 on pSMAD1/5 concentrations. Figure 6 contains a summary of this study. Studies are ongoing with ex vivo primary cardiomyocyte cultures to further investigate the potential mechanisms of developmental cardiotoxicity associated with in ovo PFOA exposure.

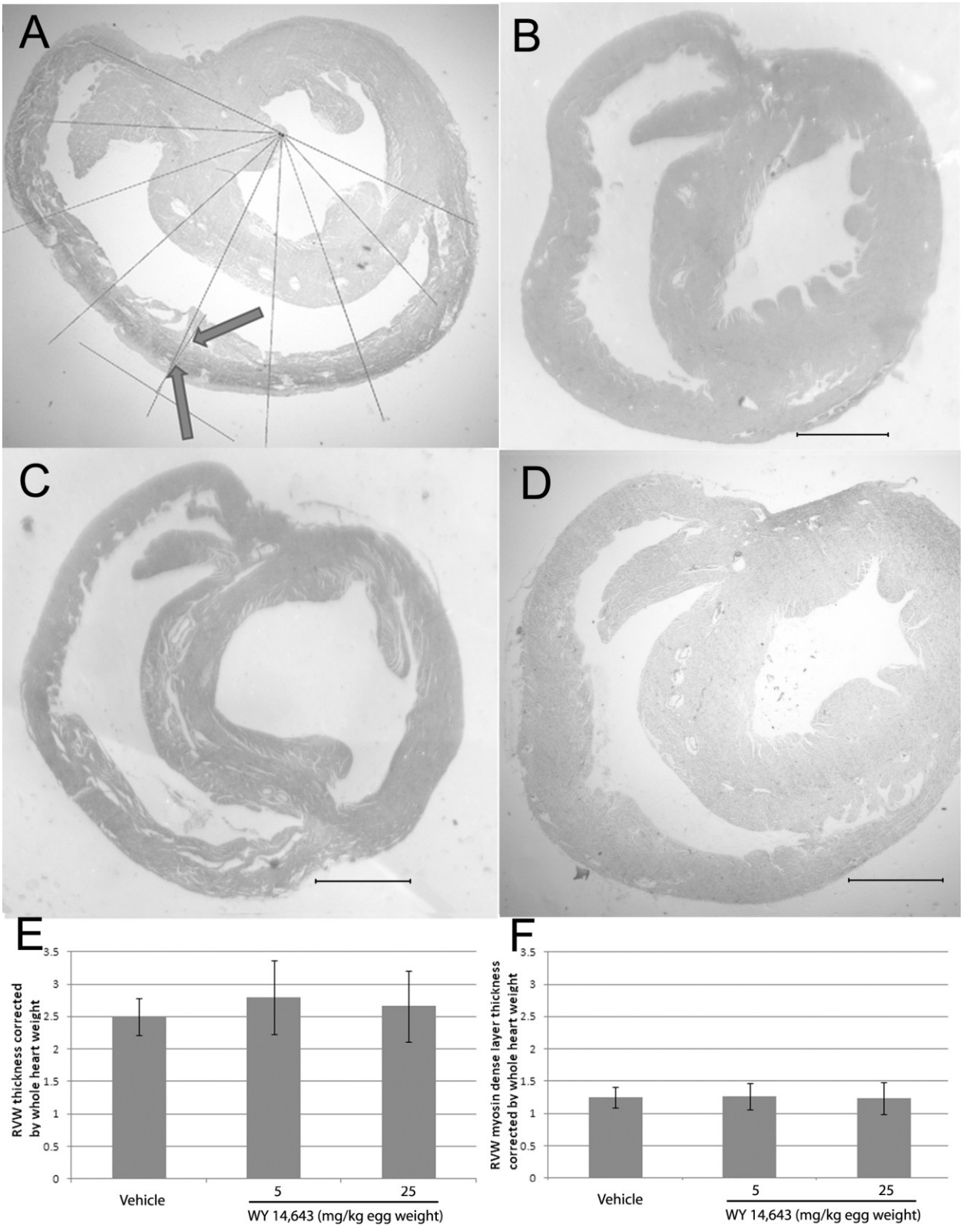
A
B
C
D
E
RVW thickness corrected by whole heart weight
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
5
25
WY 14,643 (mg/kg egg weight)
F
RVW myosin dense layer thickness corrected by whole heart weight
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
5
25
WY 14,643 (mg/kg egg weight)

Figure 3.1 Histological assessment of hearts from embryonic day 19 (ED19) chicken embryos exposed to WY 14,643 in ovo. Fertile chicken eggs were injected with 0, 5, or 25 mg of WY14,643/kg of egg weight and incubated until ED19. Hearts were sectioned at 6 μ and stained with hematoxylin and eosin or for myosin. A: Illustration of a dense layer of myosin staining, shown between arrows. B: Representative section of a heart from an ED19 chicken embryo exposed to vehicle. Scale bar represents 1000 μm. C: Representative section of a heart from an ED19 chicken embryo exposed to 2 mg of PFOA/kg of egg weight. Scale bar represents 1000 μm. D: Representative section of a heart from an ED19 chicken embryo exposed to 25 mg of WY 14,643/kg of egg weight. Scale bar represents 1000 μm. E: Quantification of mean right ventricular wall thickness. F: Quantification of mean thickness of a dense layer of myosin staining in the right ventricular wall. Error bars represent standard deviation, SD.

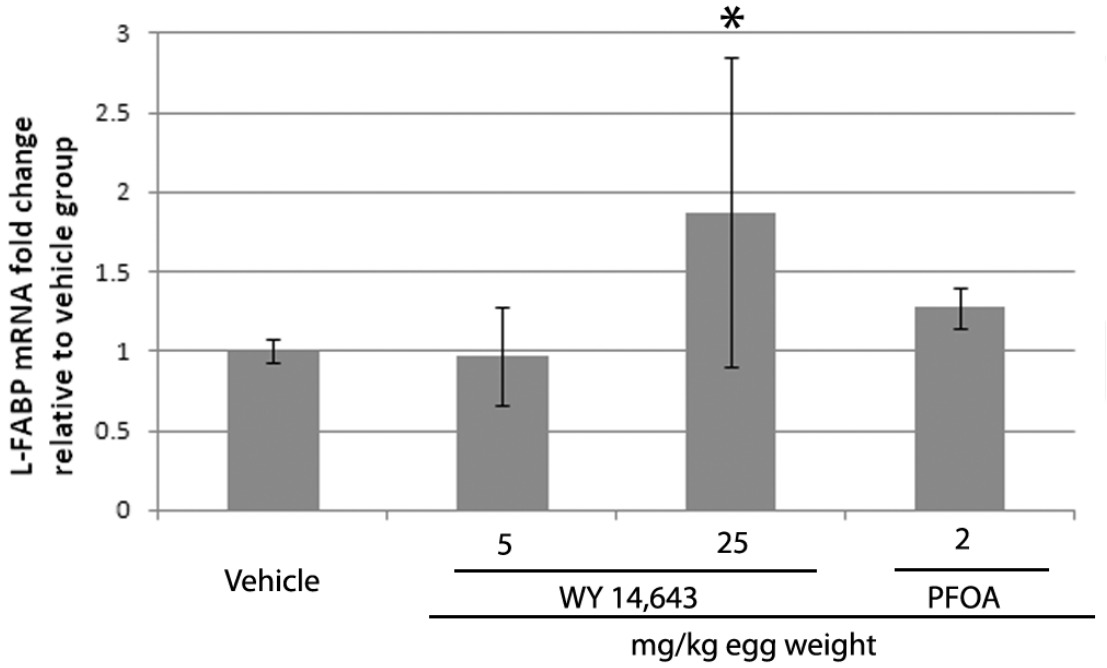

Figure 3.2 Results of quantitative real-time PCR for liver-fatty acid binding protein (L-FABP), an indicator of PPARα activation. Fertile chicken eggs were injected 0, 5, or 25 mg of WY 14,643/kg of egg weight or 2 mg of PFOA /kg of egg weight and incubated until hatch. Error bars represent standard deviation, SD. Mean expression of L-FABP in liver was assessed with PCR (N=5-6). *Statistically different from vehicle controls ($P < 0.05$).

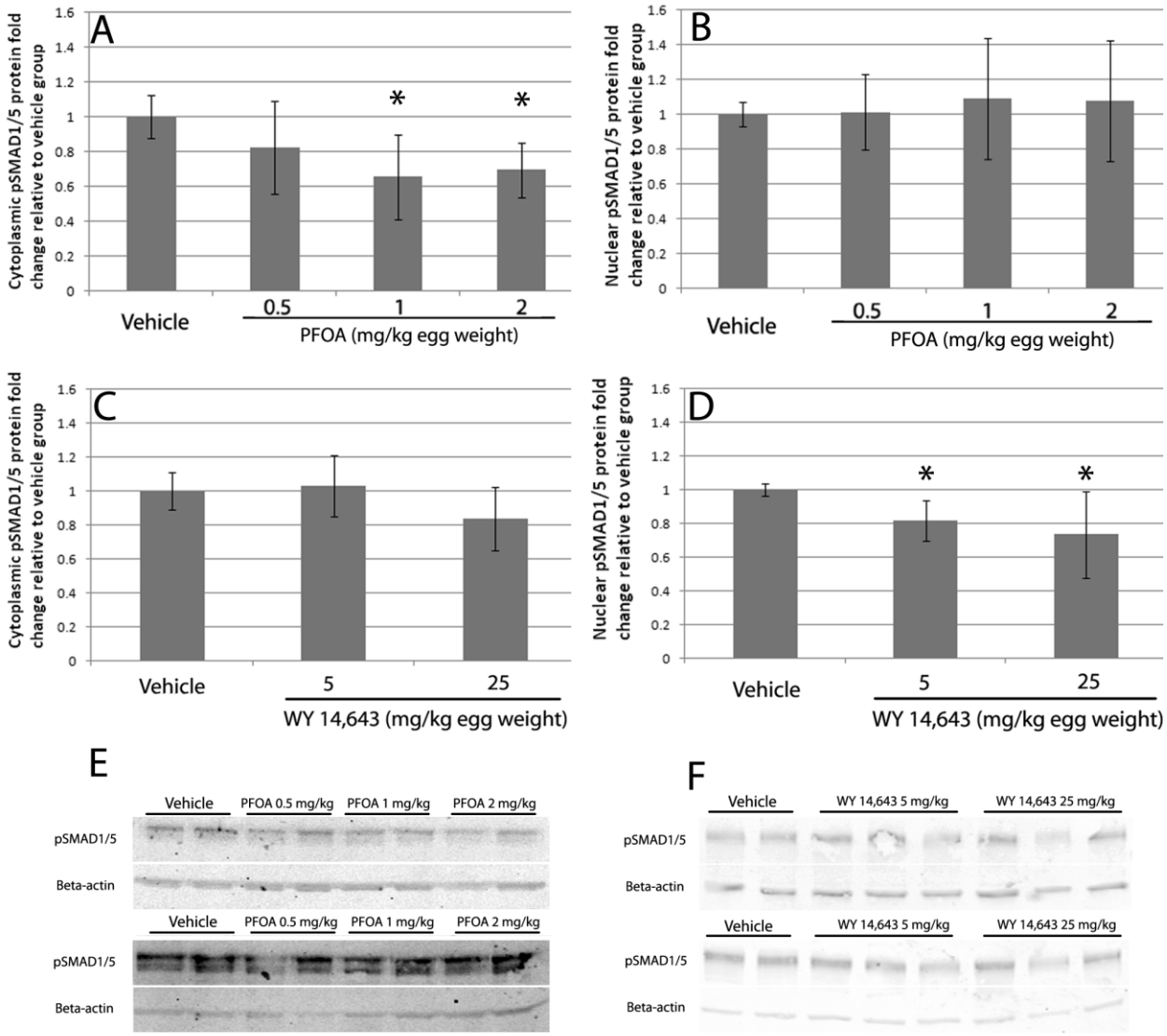

Figure 3.3 Western blot quantification of pSMAD1/5 in four-day-old (ED4) chicken embryos exposed to PFOA or WY 14,643; error bars represent standard deviation, SD. (A) pSMAD1/5 in the cytoplasmic portion of PFOA-exposed embryos (N=9-10). (B) pSMAD1/5 in the nuclear portion of PFOA-exposed embryos (N=9-10) (C) pSMAD1/5 in the cytoplasmic portion of WY 14,643-exposed embryos (N=6-10). (D) pSMAD1/5 in the nuclear portion of WY 14,643-exposed embryos (N=6-11). (E) Representative blot of pSMAD1/5 from PFOA-exposed

embryos. The upper panel is the cytoplasmic portion and the lower panel is the nuclear portion. (F) Representative blot of pSMAD1/5 from WY 14,643-exposed embryos. The upper panel is the cytoplasmic portion and the lower panel is the nuclear portion. *Statistically different from vehicle controls ($P < 0.05$).

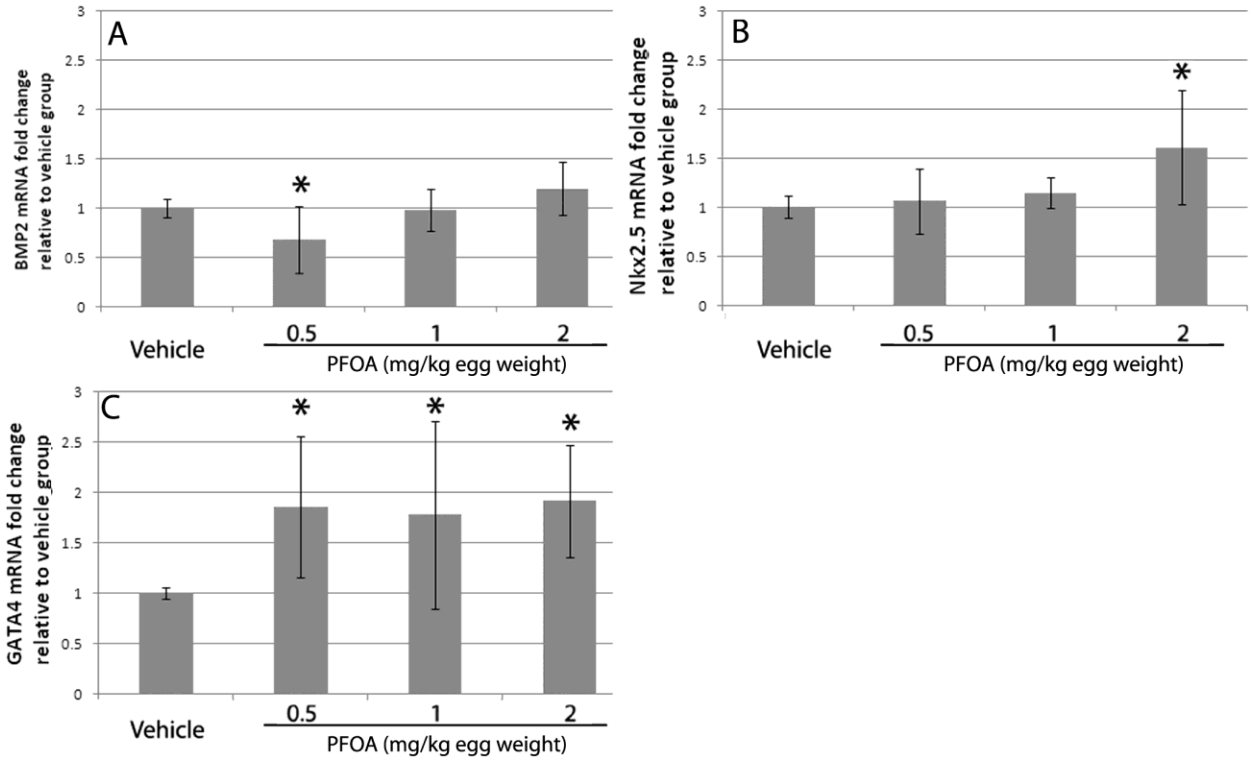

Figure 3.4 Results of quantitative real-time PCR for pathways critical in early heart development. Fertile chicken eggs were injected with 0, 0.5, 1, or 2 mg of PFOA/kg of egg weight and incubated for four (ED4) or six (ED6) days. Error bars represent standard deviation; SD. (A) BMP2 gene expression in ED4 whole embryos (N=6-9). (B) Nkx2.5 gene expression in hearts from ED6 embryos (N=8). (C) GATA4 gene expression in hearts from ED6 embryos (N=6-8). *Statistically different from vehicle controls ($P < 0.05$).

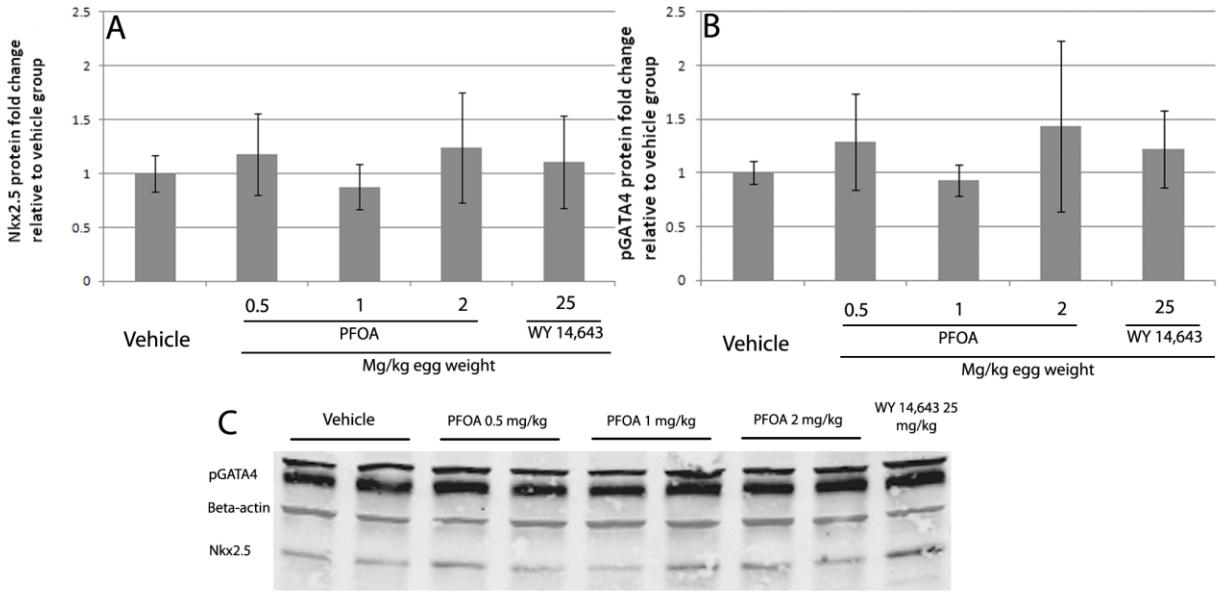

Figure 3.5 Western blot quantification of Nkx2.5 and pGATA4 in hearts from six-day-old (ED6) chicken embryos exposed to PFOA or WY 14,643. Error bars represent standard deviation, SD. (A) Nkx2.5 (N=6-10). (B) pGATA4 (N=6-8). (C) Representative blot of Nkx2.5 and pGATA4.

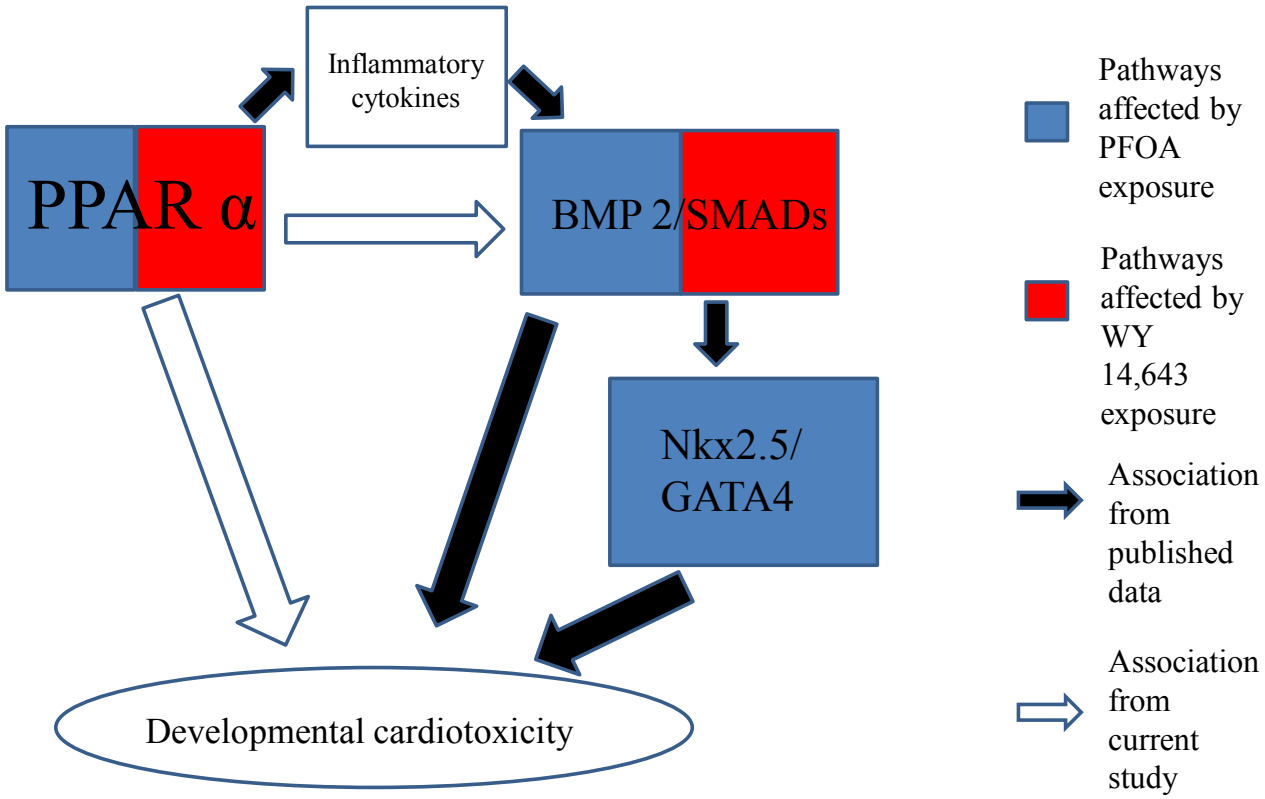

Figure 3.6 Summary of the study. Dark shaded squares represent the pathways affected by PFOA exposure; light shaded squares represent the pathways affected by WY 14,643 exposure. Closed arrows represent conclusions from published data (DeWitt et al. 2009; Luo et al. 2009; Yamane et al. 2003; Kirby 2002; Barron et al. 2000; McCulley et al. 2012; Harvey et al. 2002 and Pu et al. 2004); open arrows represent conclusions from the current study.

Table 3.1 General developmental toxicity parameters in 19-day-old chicken embryos and hatchling chickens exposed to WY 14,643 during development.

	ED19 Embryos			Hatchlings		
	Vehicle	5 mg/kg	25 mg/kg	Vehicle	5 mg/kg	25 mg/kg
Yolk-free body weight (g)	27.78±1.33	28.44±2.40	27.22±2.25	32.44±2.35	30.93±2.15	30.52±2.80
Absolute heart weight (g)	0.20±0.014	0.20±0.023	0.20±0.022	0.28±0.034	0.30±0.042	0.31±0.034
Relative heart weight	0.0072±0.00068	0.0070±0.0009	0.0074±0.00045	0.0087±0.00090	0.0096±0.00110	0.010±0.00070*
Relative liver weight	0.014±0.0024	0.013±0.0014	0.015±0.0017	0.022±0.0026	0.024±0.0029	0.025±0.0016
Mortality	20.0%	30.0%	20.0%	41.7%	25.0%	33.3%
Hatchability	-	-	-	77.8%	100.0%	100.0%

Note. Weight values are means ± standard deviation. Injection concentration was based on kg of egg weight. For organ weight, N = 7-8/dose group for ED19 embryos and 7-9/dose group for hatchlings. For mortality and hatchability, N = 11/dose group for ED19 embryos and 12/dose group for hatchlings. Relative heart and liver weight = absolute weight of each organ corrected by yolk-free body weight. Hatchability was calculated as the number of successfully hatched chickens adjusted by the number of chicken embryos that were alive at ED20. *Statistically different from vehicle control ($P < 0.05$).

Table 3.2 Mean echocardiography results of structural parameters measured in hearts of hatchling chickens exposed to WY 14,643 during development.

Parameter	Value		
	Vehicle	5 mg/kg	25 mg/kg
Left ventricular wall posterior dimension at diastole (mm)	0.506±0.009	0.615±0.027*	0.996±0.031*
Left ventricular wall posterior dimension systole (mm)	1.188±0.006	1.436±0.019*	1.712±0.168*
Left ventricular volume at diastole (ul)	70.055±6.879	59.902±6.322*	44.796±4.175*
Left ventricular volume at systole (ul)	15.136±1.862	8.203±1.873*	4.196±1.322*
Left ventricule mass (mg)	93.3±5.0	115.6±6.3*	134.8±4.0*
Heart rate (bpm)	246.0±18.1	364.0±18.4*	393.0±7.9*
Stroke volume (ul)	54.919±8.332	51.699±6.887	40.600±5.033*
Cardiac output (ml/min)	192.2±19.0	313.6±15.9*	355.4±16.5*
Ejection fraction (%)	78.1±4.3	86.1±3.6*	90.4±3.7*
Fractional shortening (%)	58.6±1.2	56.8±2.6	62.2±4.1*

Note. Values are means ± standard deviation. Injection concentration was based on kg of egg weight. N = 7–9/dose group. *Statistically different from vehicle controls ($P < 0.05$).

CHAPTER 4 PFOA-INDUCED TOXICITY IN PRIMARY CULTURES OF CHICKEN EMBRYO CARDIOMYOCYTES (WILL BE SUBMITTED TO ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY)

Abstract

Perfluorooctanoic acid (PFOA) is a widespread environmental contaminant that induces developmental cardiotoxicity in late stage chicken embryos and hatchling chickens. To investigate mechanism(s) of cardiotoxicity, primary cultures of cardiomyocytes were prepared from ten-day-old chicken embryos that were A) pre-exposed to vehicle or 2 mg of PFOA/kg of egg weight in ovo or B) incubated with PFOA in vitro at concentrations ranging from 0 to 100 μg/ml in medium for one or 36 hours. . When viability was assessed, survival of cardiomyocytes prepared from pre-exposed embryos did not differ from vehicle controls, even under conditions of serum starvation designed to challenge the cells. One hour of exposure to 100 μg/ml of PFOA in vitro and 36 hours of exposure to 75 and 100 μg/ml PFOA in vitro decreased viability. When contractility was evaluated, cardiomyocytes cultured from pre-exposed embryos exhibited decreases in time to maximum departure velocity and cell length at peak contraction, whereas cardiomyocytes exposed in vitro exhibited a reduction in the 50% relaxation time at a concentration of 1 μg/ml relative to vehicle controls. Reactive oxygen species (ROS) generation, which was evaluated only in cultures exposed to PFOA in vitro, was significantly induced following incubation with 50 μg/ml of PFOA for one hour. These data indicate that while in vitro exposure to relatively high concentrations of PFOA can induce cytotoxicity and ROS, developmental cardiotoxicity observed in ovo is not likely mediated via PFOA-induced overt cytotoxicity, but by interference of upstream signals that regulate early heart development.

4.1 Introduction

Perfluorooctanoic acid (PFOA) is a fluorinated compound that belongs to the perfluoroalkyl acid (PFAA) family of chemicals. Production of PFOA began in the 1950s and it is primarily used as a polymerization aid in the manufacture of fluorinated polymers and elastomers for myriad industrial and consumer products. PFOA is persistent in environmental media (Vaalgamaa et al. 2011) and is now detected in biota as well as the serum of the general human population. Between 2009 and 2010 in the US, the median serum concentration in the general human population was 3.2 ng/ml (CDC 2012). However, it is estimated that exposure levels could be much higher in areas with contaminated media. For example, Shin et al. (2011) reported a median serum value of 24.3 ng/ml in residents from an area of high contamination in West Virginia and Ohio, US. Hoffman et al. (2011) reported a median serum value of 75.7 ng/ml in communities surrounding a fluoropolymer production facility. The highest serum levels were observed in occupationally-exposed workers; Olsen et al. (2007) reported up to 5100 ng/ml serum. Due to increasing reports of adverse health effects, the US Environmental Protection Agency (USEPA) worked with eight major manufacturers of PFOA to establish a stewardship program that will phase out PFOA in emissions and products in the US by 2015 (USEPA 2013). However, the stability of PFOA in the environment and its long half-life in humans will likely mean that exposure will continue for a considerable time.

There is epidemiological evidence that PFOA affects human development. Stein et al. (2009) reported an association of PFOA serum concentrations with preeclampsia and birth defects. Frisbee et al. (2010) reported association between PFOA exposure and elevated serum cholesterol levels and uric acid levels in children and adolescents. A recent study correlates

PFOA exposure to decreased vaccination responses in children between five and seven years old living in the Faroe Islands (Grandjean et al. 2012).

In animal models, PFOA is known to induce hepatic, pancreatic, and testicular cancers (Biegel et al. 2001), endocrine disruption (Olsen et al. 1998), immunotoxicity (DeWitt et al. 2008), and developmental toxicity, including increased mortality and retarded development (Wolf et al. 1997). The mechanisms underlying high mortality and retarded development following developmental exposure to PFOA have not yet been elucidated.

In our previous study (Jiang et al. 2012), we observed developmental cardiotoxicity in a chicken model. Following developmental exposure to PFOA, chicken embryo hearts exhibited morphological changes, including thinning of the right ventricular wall and thinning of a layer of dense myosin staining within the right ventricular wall. Hearts from developmentally-exposed hatchling chickens had increased left ventricular dimension and altered left ventricular function. In this study, we further explored developmental cardiotoxicity associated with PFOA exposure by evaluating changes in primary cardiomyocytes cultured from embryonic day 10 chickens. Viability, contractility, and reactive oxygen species (ROS) generation were evaluated to assess PFOA-induced cytotoxicity.

4.2 Materials and methods

4.2.1 Animals

Fertile chicken (*Gallus gallus*) eggs were purchased from the North Carolina State University Poultry Research Center (Raleigh, NC). Eggs were cleaned with 20% povidone iodine, weighed, given ID numbers, and evenly distributed by weight among doses. The eggs used for air cell injections were candled to outline air cells in pencil on shells.

4.2.2 Chemicals

Sunflower oil was purchased from Spectrum Organic Products, LLC (Boulder, CO). PFOA and other chemicals (if not otherwise mentioned) were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.3 Egg injection, incubation and sample collection

For primary cardiomyocyte cultures prepared from pre-exposed embryos, egg injection and incubation were carried out as described previously (Jiang et al. 2012). Briefly, PFOA was suspended in sunflower oil and vortexed before injection into each egg. Stock solutions were prepared so that 0.1 µl of solution per gram of egg resulted in the appropriate mg/kg concentration. Dose selection for PFOA was based on observations by O'Brien et al. (2009) and data from our previous work (Jiang et al. 2012). O'Brien et al. reported that up to 10 mg/kg of a PFOA injection does not affect pipping success; in our previous studies, injection of 2 mg of PFOA/kg of egg weight resulted in a serum concentration of 5670 ng/ml in hatchlings, a level comparable to occupationally-exposed humans.

Uninjected eggs (for preparation of primary cardiomyocyte cultures exposed in vitro) and injected eggs (for preparation of pre-exposed primary cardiomocyte cultures) were incubated for ten days in a Lyon Roll-X incubator (Chula Vista, CA) set at 99.5–100ºF dry bulb and 87–88º F wet bulb. Eggs were incubated in separate batches to avoid cross-contamination and the incubator was cleaned after each round of incubation. At ED10, eggs were removed from the incubator and opened under sterile conditions in a cell culture hood. Embryos were removed from eggs and hearts were removed and dissected, and treated as described in the following section. All procedures were approved by the East Carolina University Institutional Animal Care and Use Committee.

4.2.4 Primary cardiomyocyte cultures

The primary cardiomyocyte culture procedure was as described in Jones et al. (2009) with minor modifications. Briefly, hearts were rinsed in cardiomyocyte medium (54% Hanks balanced salt solution (HBSS), 40% medium M199, 6% fetal bovine serum, 100 unit/ml penicillin, 0.1 mg/ml streptomycin, and 0.03mg/ml 5-bromo-2-deoxyouridine (BrdU)). A pre-digestion was carried out with digestion buffer (0.5 mg/ml trypsin and 0.05 mg/ml DNAse I (Roche, Mannheim, Germany) in HBSS) in a shaking incubator at 37°C for ten minutes. The resulting suspension was discarded and five to seven more digestion cycles were performed in digestion buffer in a shaking incubator at 37°C for seven minutes each cycle. The same volume of ice cold trypsin inhibitor buffer (10 mg/ml albumin, 20% fetal bovine serum, 80% HBSS with antibiotics) was then mixed with the final suspension and filtered through a filtration apparatus (BioDesign Inc. of New York, NY). The suspension was centrifuged, washed, and resuspended in cardiomyocyte medium at 1:32 dilution (mg/µl). The stock cell suspension was plated in 96-well plates at 70-80% coverage (33.3 µl stock/cm2 , 1:20 concentration in medium) for viability and ROS detection assays, or plated in 6-well plates at 30-50% coverage (10.4 µl stock/cm2, 1:20 concentration in medium) for contractility assays. After 24 hours, the cells were ready for treatment and/or evaluation as by that time they had adhered to the plate and exhibited spontaneous beating (at 70-80% coverage) that was observable under an inverted microscope. Figure 4.1 shows a representative image of primary cardiomyocytes after 72h of incubation.

4.2.4.1 Primary cardiomyocyte cultures from pre-exposed embryos

To determine *developmental* effects of PFOA on cardiomyocytes, groups of eggs were injected with either vehicle or 2 mg of PFOA/kg of egg weight prior to incubation. Freshly isolated cells from injected eggs were plated in 96-well culture plates, as described above, in

triplicate. After 24 hours, attached cardiomyocytes were serum-starved to evaluate the effects of stress on cell survival. Cells were treated with normal medium, low serum medium (1.8% serum), serum free medium (0% serum), or no medium as a positive control (HBSS with antibiotics and BrdU). Cells were cultured for 0, 12, or 24 hours and viability was assessed with a Celltiter 96 AQueous One Solution Cell Proliferation Assay (MTS) viability kit (Promega, Madison, WI), using the manufacturer's protocol. To evaluate contractility, a separate group of cells isolated from ED10 embryos pre-exposed to PFOA in ovo were plated into 6-well culture plates containing 25 mm round glass coverslips and cardiomyocyte medium and incubated. After 24 hours, the glass coverslips were removed from the plates, rinsed in Tyrode buffer (sodium chloride 137 mM, potassium chloride 5.4 mM, calcium chloride 1.2 mM, magnesium chloride 1.5 mM, HEPES 10 mM, and glucose 10mM), and installed on a stimulation chamber (IonOptix, Milton, MA) in an IonOptix system (Milton, MA). An IonOptix MyoPacer Cell Stimulator was used to stimulate the cells at 20V, 1HZ, at room temperature. Departure velocity, time to departure velocity, time to 50% peak contraction, time to 50% relaxation, cell length at maximum contraction, and cell shortening percentage at peak contraction were measured.

4.2.4.2 Primary cardiomyocyte cultures exposed to PFOA in vitro

To determine *direct* effects of PFOA on cardiomyocytes, three separate cultures of primary cardiomyocytes were prepared from ED10 embryos collected from uninjected eggs. For evaluating viability, cultures were incubated for 24 hours and then exposed to vehicle (cardiomyocyte medium with 0.1% DMSO) or 0.1, 1, 10, 50, 75, or 100 µg/ml of PFOA in cardiomyocyte medium with 0.1% DMSO for one or 36 hours. At least three independent isolations were used for each experimental group. Viability was assessed with a Celltiter 96

AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI), using the manufacturer's protocol.

To evaluate contractility, cultures were plated into 6-well culture plates containing 25 mm round glass coverslips and cardiomyocyte medium and incubated. After 24-26 hours, cultures were exposed to vehicle (cardiomyocyte medium with 0.1% DMSO) or 0.1, 1, 10, 50, 75, or 100 μg/ml of PFOA in cardiomyocyte medium with 0.1% DMSO for one hour. The glass coverslips were then removed from the plates and contractility was evaluated with IonOptix instruments as described above.

To evaluate ROS, cultures were incubated for 24 hours and then 2',7'-dichlorofluorescein (DCF; Life technologies, Grand Island, NY) stock solution made with Dulbecco's phosphate-buffered saline (DPBS) and DMSO was added for a final concentration of 20 μM in the medium. After incubating cultures for 30 minutes in DCF, cultures were exposed to vehicle (cardiomyocyte medium with 0.1% DMSO) or 0.1, 1, 10, or 50 μg/ml of PFOA in cardiomyocyte medium with 0.1% DMSO for one more hour. The medium was removed, cells were washed twice in PBS, and plates were read at excitation 495nm/emission 525nm (Tecan Infinite M200Pro, Research Triangle Park, NC) to detect ROS. Values were normalized to the vehicle treated group for fold changes.

4.2.5 Statistical analysis

Data are presented as mean ± standard deviation (SD) for both cell viability and ROS. Contractility data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with the SAS JMP System (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was used to determine dose-dependent effects for all in vitro endpoints and for contractility results of the cultures prepared from pre-exposed embryos. When ANOVA

results indicated statistically significant dose effects, individual post hoc comparisons were made with a two-tailed t-test. Two-way ANOVA was used to determine dose and serum concentration effects on ex vivo viability. When ANOVA results indicated statistically significant main effects of dose and serum concentration without interactions between dose and serum concentration, individual post hoc comparisons were made with a two-tailed t-test. Statistical significance was determined when P-values were less than 0.05.

4.3 Results

4.3.1 Primary cardiomyocyte cultures from pre-exposed embryos

Two-way ANOVA indicated that serum starvation impacted cell viability in cardiomyoctyes from both control and PFOA-exposed embryos. PFOA exposure itself did not impact cell viability (Figure 4.2). Among the contractility parameters measured with the IonOptix System, the time to departure velocity was decreased by 40.9% in cardiomyocytes from PFOA-exposed embryos relative to controls (Figure 4.3b). The cell length at maximum contraction was decreased by 23.7% in cardiomyocytes from PFOA-exposed embryos relative to controls (Figure 4.3e). No statistical differences were detected for other parameters (Figures 4.3a, c, d and f).

4.3.2 Primary cardiomyocyte cultures exposed to PFOA in vitro

Following one hour of exposure to 100 µg/ml of PFOA, cell viability was decreased by 72.4% (Figure 4.4a). After 36 hours of exposure to75 or 100 µg/ml of PFOA in cardiomyocyte medium, viability decreased by 26.4% and 69.4%, respectively (Figure 4.4b). Among the contractility parameters measured with the IonOptix System, the time to 50% relaxation was increased by 38.2% following one hour of exposure to 1 µg/ml of PFOA in cardiomyocyte

medium (Figure 4.5d). No significant differences were found for other parameters measured (Figure 4.5a, b, c, e and f). One hour of exposure to 50 μg/ml of PFOA in cardiomyocyte medium increased ROS generation in primary cardiomyocytes by 316.8% (Figure 4.6).

4.4 Discussion

In our previous study (Jiang et al. 2012), developmental cardiotoxicity was observed in chicken embryos and hatchling chickens exposed to PFOA via air cell injection prior to incubation. Hearts of PFOA-exposed ED19 embryos had thinner right ventricular walls relative to control embryos. When hearts of the hatchling chickens were evaluated with echocardiography, left ventricle hypertrophy and elevated functional parameters were observed in exposed hatchlings relative to control hatchlings. We undertook the current study to explore potential cellular mechanisms of PFOA-induced developmental cardiotoxicity. Our experiments were designed to assess whether the developmental cardiotoxicity was a result of direct cytotoxicity of PFOA to cardiomyocytes or a result of effects on upstream developmental processes. The concentration of PFOA injected into chicken eggs prior to incubation was 2 mg/kg of egg weight, the highest dose that was injected in our previous study for characterization of PFOA-induced developmental cardiotoxicity (Jiang et al. 2012). This dose is approximately 2 μg/ml if perfect equilibrium is achieved within eggs. The average PFOA serum concentration in hatchling chickens from eggs injected with 2 mg/kg prior to incubation was 5.67 μg/ml (Jiang et al. 2012). Although the precise dose at a specific time point during development was not determined, the actual exposure to the developing embryos was likely between 2 μg/ml and 5.67 μg/ml. The PFOA concentrations used in the in vitro experiments were 0.1, 1, 10, 50, 75 and 100 μg/ml. Slotkin et al. (2008) demonstrated that at concentrations below100 μg/ml of PFOA, cell

damage in PC-12 cells was not detectable. Our range of concentrations for the in vitro experiments therefore included low concentrations (0.1 μg/ml), a concentration close to the ex vivo dose (1 μg/ml), and higher concentrations to cover a wide range. A concentration of 50 μg/ml was used as the highest dose for the contractility and ROS generation assays to ensure that observed affects were not attributable to overt cytotoxicity, as evaluation of viability indicated that 75 μg/ml and 100 μg/ml induced cell death.

PFOA did not markedly affect the yield of cells by weight per embryo heart (data not shown) or reduce viability of primary cardiomyocytes prepared from pre-exposed embryos, either under normal conditions or conditions of serum starvation. The serum starvation conditions were applied to investigate the ability of cardiomyocytes developmentally exposed to PFOA to survive under stressful conditions. This particular challenge was chosen as toxicity of PFOA is associated with activation of the peroxisome proliferator activated receptor alpha (PPARα), a transcription factor that regulates fatty acid and other forms of energy metabolism (Montanez et al. 2012). Our results demonstrated that pre-exposure of embryos to 2 mg/kg of PFOA did not significantly impact the ability of the cardiomyocytes to survive an energy challenge. Alternatively, when primary cardiomyocytes were exposed to PFOA in vitro, viability was decreased, but only at the highest concentrations (75 and 100 μg/ml). These concentrations far exceed the dose applied to the pre-exposed cardiomyocytes and our previous in ovo study (Jiang et al. 2012). While extreme conditions associated with very high exposure levels may induce cell death, overt cytotoxicity is likely not a key mechanism of PFOA-induced developmental cardiotoxicity. These results suggest that decreased cell viability or a potential deficiency in processes associated with energy metabolism likely do not significantly contribute to cardiotoxicity arising from developmental PFOA exposure.

Assessing contractility of cardiomyocytes provided information about the ability of cardiomyocytes from ten-day-old chicken embryos to function normally. At ED10, cardiomyocytes are well developed, capable of spontaneous beating in culture (Bézie et al. 1996) and respond to an external pacer. In the primary cardiomyocyte cultures from pre-exposed embryos, the time to reach maximum departure velocity (the maximal rate of cell edge position change during the contraction) was significantly decreased. This finding is consistent with our previous echocardiography data that recorded an increased heart rate in hatchling chickens exposed to PFOA throughout embryonic development (Jiang et al. 2012). While the cell shortening percentage at maximum contraction, which is equivalent to fractional shortening at the cellular level, did not differ significantly between vehicle-exposed and PFOA-exposed cells, the cell length at peak contraction was decreased. This finding is related to the increased ejection fraction, fractional shortening, and decreased left ventricular volume observed in hatchling chickens exposed to PFOA throughout embryonic development (Jiang et al. 2012). As an optimized cell shape is required for normal contractility (Kuo et al. 2012), an abnormal cell length could indicate that structural compensation is necessary to maintain the same degree of cardiac output. Long-term compensation therefore could eventually lead to cardiac remodeling and heart failure (Calvé et al. 2012). These data lead us to hypothesize that PFOA affects cardiomyocyte development via interference of early stage cardiomyocyte differentiation and/or proliferation.

Cells that were directly exposed to PFOA in vitro for one hour did not show similar alterations. The functional parameter that was significantly changed was an increase in time to 50% relaxation following 1 μg/ml of PFOA exposure. Changes to this parameter indicate slower repolarlization and thus a potentially longer QT interval, a common indicator of cardiotoxicity

typically induced by drugs (Recanatini et al. 2005). Additionally, a slower repolarization will potentially lead to a slower heart rate, which is contradictory to the increase in heart rate that we observed in PFOA-exposed hatchling chickens (Jiang et al. 2012). Although 1 μg/ml of PFOA in vitro did not statistically decrease cell viability, it is possible that this concentration of PFOA was sufficient to impact the ability of the cardiomyocytes to function optimally. Interestingly, this dose is close to the PFOA serum levels measured in hatchling chickens exposed in ovo (Jiang et al. 2012). The precise reason for a lack of detectable effects at the 10 and 50 μg/ml concentrations is unknown. The relatively large variability associated with evaluating contractility in the cardiomyocytes might have prevented the changes from being detected. While the change in time to 50% relaxation indicates that PFOA can impact cardiomyocytes exposed in vitro, it does not seem to be a mechanism for the developmental cardiotoxicity that we observed in ovo in Jiang et al. (2012) as this functional change is somewhat inconsistent with the in ovo results. The animals exposed in ovo had a higher heart rate and a slower relaxation will likely lead to slower heart rate. Our mechanistic work (Jiang et al. under review) indicated that PPARα is partially involved in PFOA-induced developmental cardiotoxicity in ovo as animals exposed to a PPARα agonist had similar functional, but not histological changes to the heart. We therefore speculate that PPARα agonism also is not mediating this particular effect as PPARα and PPARγ activation does not induce a long QT interval in human patients (Sturm et al. 2012).

In summary, the primary cardiomyocytes isolated from hearts ED10 chicken embryos pre-exposed to 2 mg of PFOA/kg of egg weight had functional and morphological changes that were consistent with and support our previous in ovo findings. The differences between effects observed in primary cardiomyocytes pre-exposed to PFOA or directly exposed in vitro suggest that mechanisms of developmental cardiotoxicity induced by PFOA are mediated via upstream

interference of early differentiation and/or proliferation of cardiomyocytes, not via overt cytotoxicity of PFOA to cardiomyocytes.

ROS generation is a main mechanism of direct cytotoxicity. Cardiomyocytes are known to be vulnerable to ROS generation, which is the molecular mechanism of cardiotoxicants such as doxorubicin (Ludke et al. 2012). PFOA has been reported to induce ROS generation (Eriksen et al. 2010); however, we were unable to uncover published results on ROS generation in cardiomyocytes following PFOA exposure. Cells that were exposed to PFOA in vitro for one hour after a 24-hour incubation had a significant increase in ROS generation, but only at the highest concentration tested (50 µg/ml). This suggests that ROS generation could contribute to developmental cardiotoxicity but only at high doses. Therefore, ROS generation in cardiomyocytes does not likely contribute substantially to the cardiotoxicity observed in hatchling chickens exposed to PFOA throughout embryonic development (Jiang et al. 2012).

4.5 Conclusion

In this study, decreased cell viability and increased ROS generation were observed in primary cardiomyocytes that were directly exposed to PFOA in vitro, but only at high concentrations. Therefore, overt cytotoxicity induced by PFOA is unlikely unless exposure levels are very high, implying that this mechanism is not relevant to human health except at very high exposures. For example, Olsen et al. (2007) reported occupational exposures that resulted in PFOA serum concentration of 5100 ng/ml, which is approximately equivalent to 5.1 µg/ml in vitro. Direct exposure of cardiomyocytes to PFOA in vitro also increased relaxation time for contraction, suggesting that PFOA may induce a long QT interval. Additional work is necessary to determine if this one functional change is relevant to public health.

Altered morphology and function were observed in cardiomyocytes isolated from pre-exposed embryos and this positive chronotropic effect is consistent with our previous in ovo findings. Directly-exposed cardiomyocytes did not have similar alterations after one hour of in vitro exposure to PFOA, suggesting that PFOA induces developmental cardiotoxicity in early stages of development, not through direct contact with developed cardiomycoytes. These morphological and functional alterations occur at relatively low doses that are comparable with concentrations observed in the general human population. Further work is necessary to further elucidate the molecular mechanisms of PFOA interference with early heart development.

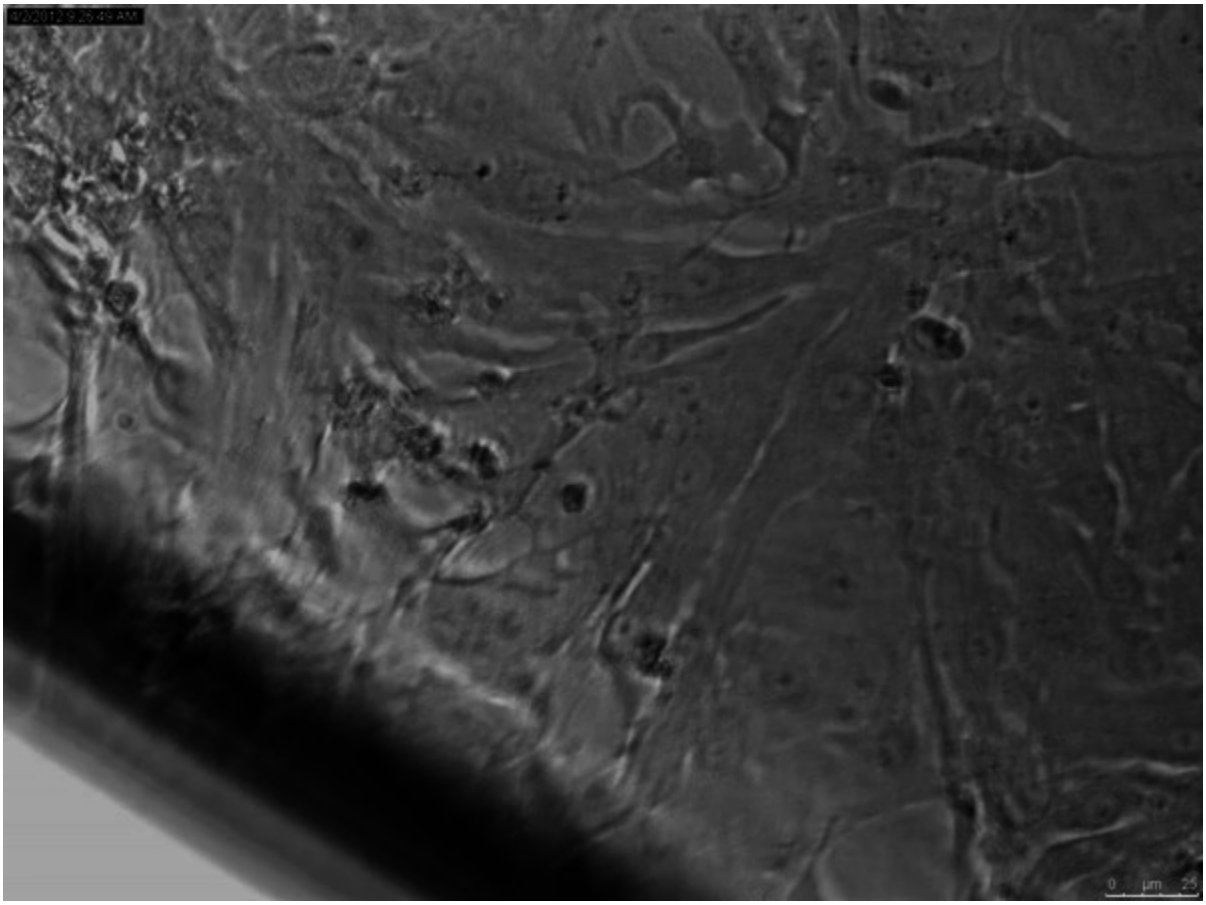

Figure 4.1 Representative image of primary cardiomyocyte isolated from ED10 chicken embryo hearts incubated for approximately 72 hours.

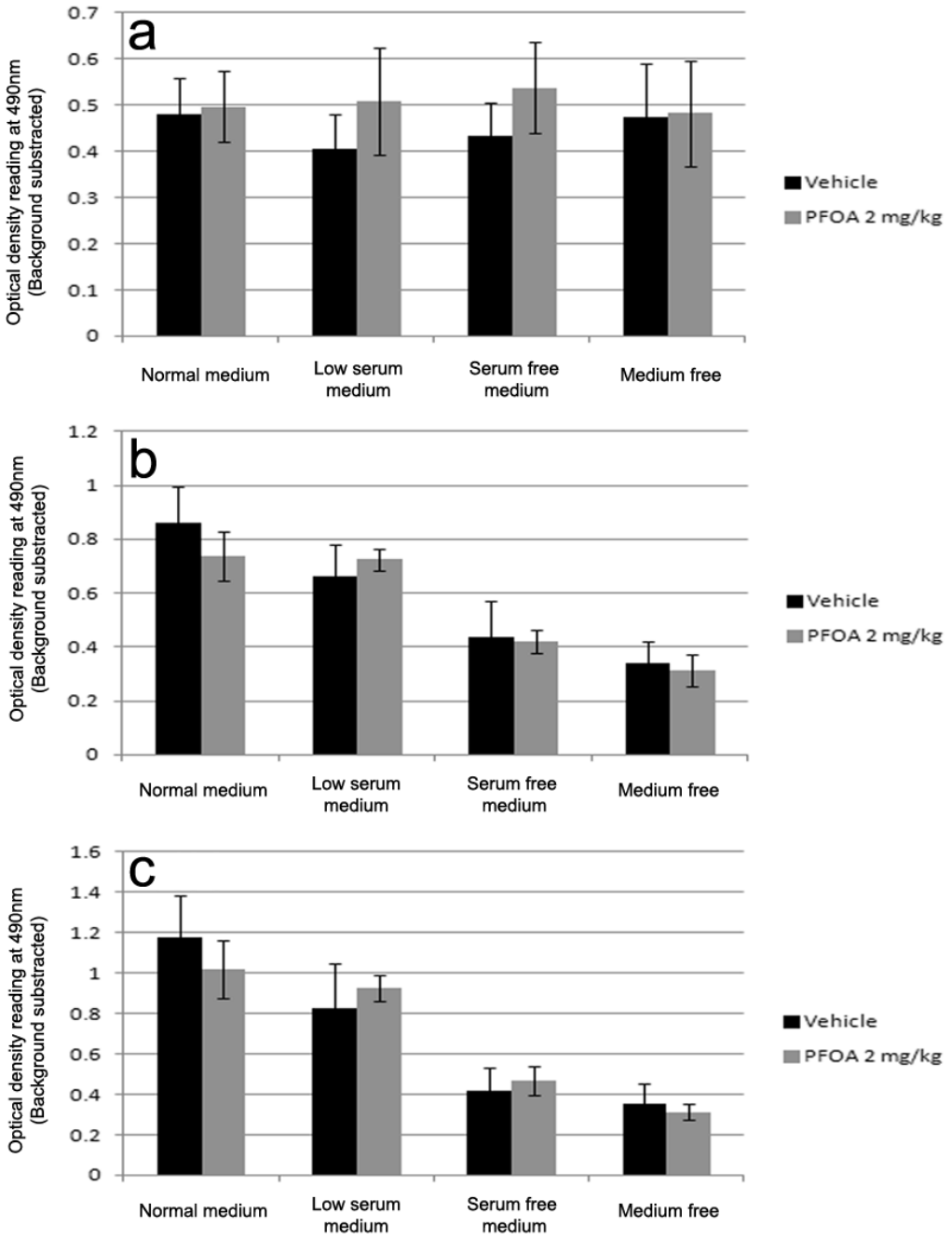

Figure 4.2 Cell viability of primary cardiomyocytes exposed to vehicle or 2 mg of PFOA/kg of egg weight prior to incubation. Air-cell injected fertile chicken eggs were incubated until embryonic day ten and primary cardiomyocytes were isolated from embryos. Three independent isolations of primary cardiomyocytes were used for both exposure groups. Following 24 hours of incubation, cardiomyocytes were exposed to normal medium, low serum medium, serum-free

medium or medium-free conditions for 0, 12, or 24 hours. (a) Viability of primary cardiomyocytes subjected to brief serum starvation (0 hours) (N=3-4). (b) Viability of primary cardiomyocyte subjected to serum starvation for 12 hours (N=3-4). (c) Viability of primary cardiomyocyte subjected to serum starvation for 24 hours (N=3-4). Data are presented as mean ± SD.

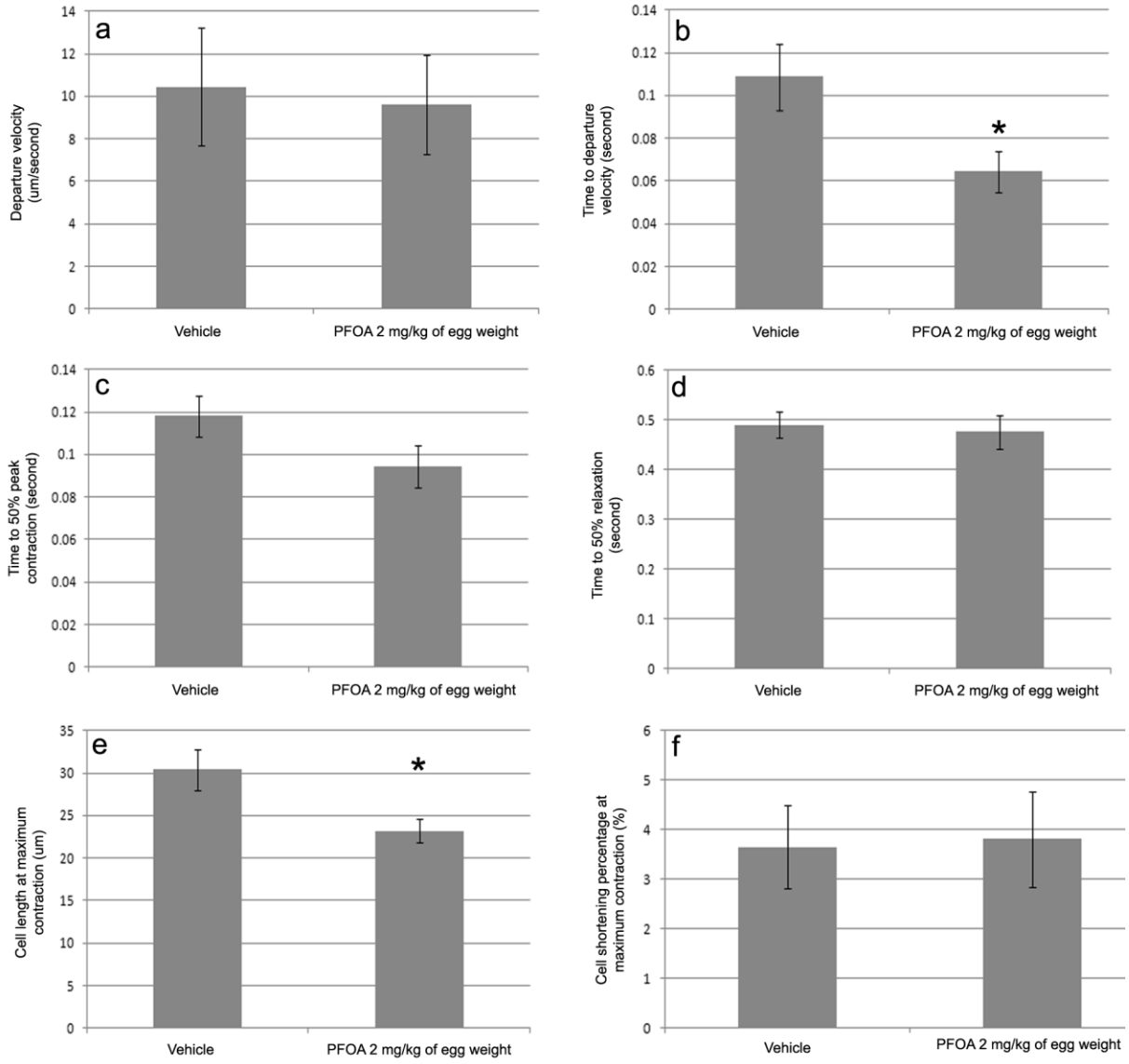

Figure 4.3 Contractility of primary cardiomyocytes exposed to vehicle or 2 mg of PFOA/kg of egg weight prior to incubation. Three independent isolations of primary cardiomyocytes were used for both exposure groups. Air-cell injected fertile chicken eggs were incubated until embryonic day ten; primary cardiomyocytes were isolated from embryos and incubated for 24 hours. (a) Departure velocity (N=30 for vehicle, 29 for PFOA-exposed). (b) Time to departure

velocity (N=30 for vehicle, 29 for PFOA-exposed). (c) Time to 50% peak contraction (N=30 for vehicle, 29 for PFOA-exposed). (d) Time to 50% relaxation (N=29 for vehicle, 28 for PFOA - exposed). (e) Cell length at maximum contraction (N=30 for vehicle, 29 for PFOA-exposed). (f) Cell shortening percentage at maximum contraction (N=30 for vehicle, 29 for PFOA-exposed). Data are presented as mean ± SEM. * Denotes statistical difference from vehicle group ($P<0.05$).

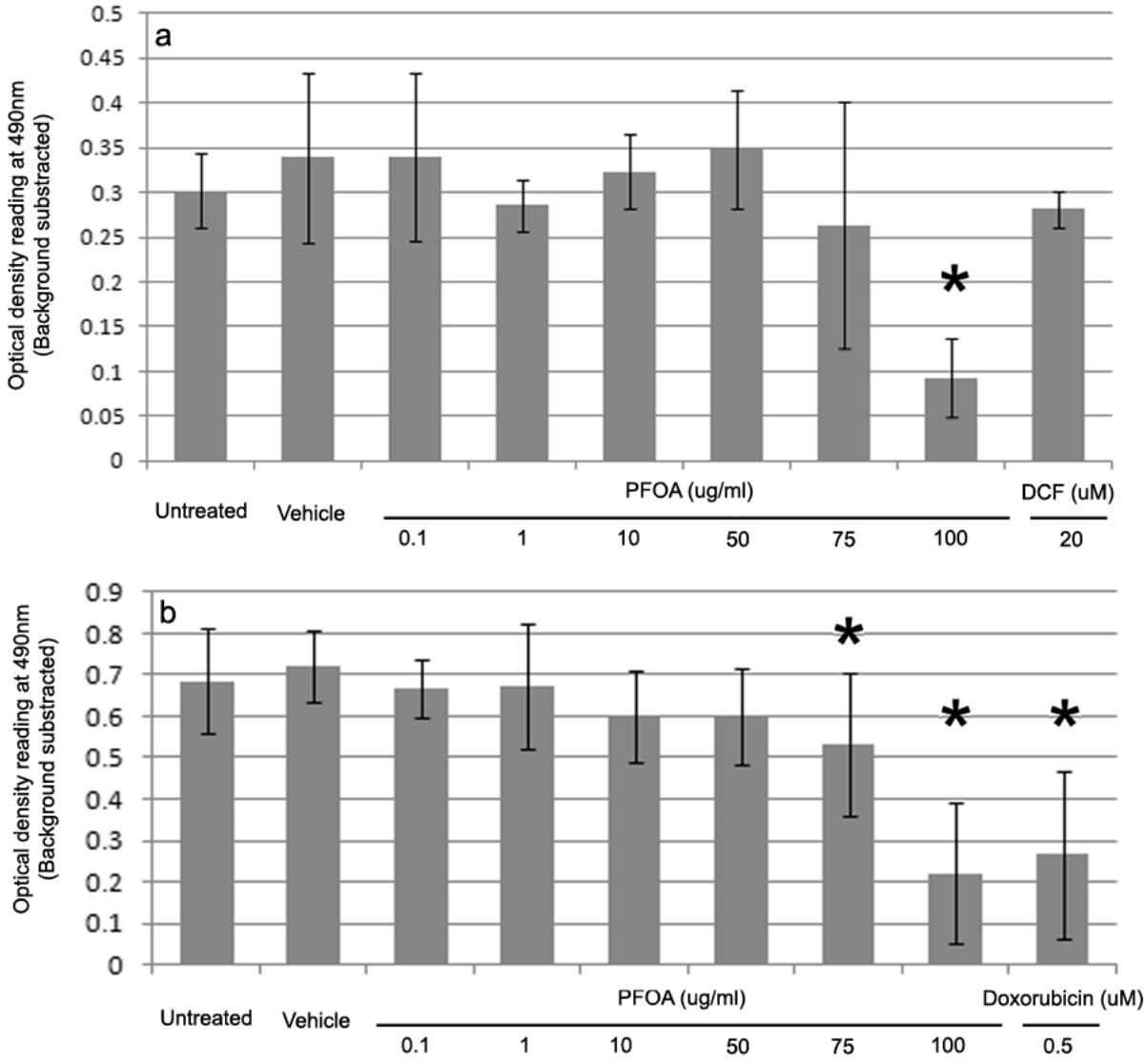

Figure 4.4 Cell viability of primary cardiomyocytes exposed to PFOA in vitro. Unexposed fertile chicken eggs were incubated until embryonic day ten and primary cardiomyocytes were isolated from embryos. Three independent isolations of primary cardiomyocytes were used. Following 24 hours of incubation, cardiomyocytes were exposed to 0, 0.1, 1, 10, 50, 75 or 100 µg/ml PFOA in 0.1% DMSO and incubated for one or 36 hours. Doxorubicin applied at 0.5 uM

was included as positive control in the 36-hour exposure group. (a) Viability of primary cardiomyocytes exposed to PFOA for one hour (N=4-6). (b) Viability of primary cardiomyocytes exposed to PFOA for 36 hours (N=4-6). Data are presented as mean ± SD. * Denotes statistical difference from vehicle group ($P<0.05$).

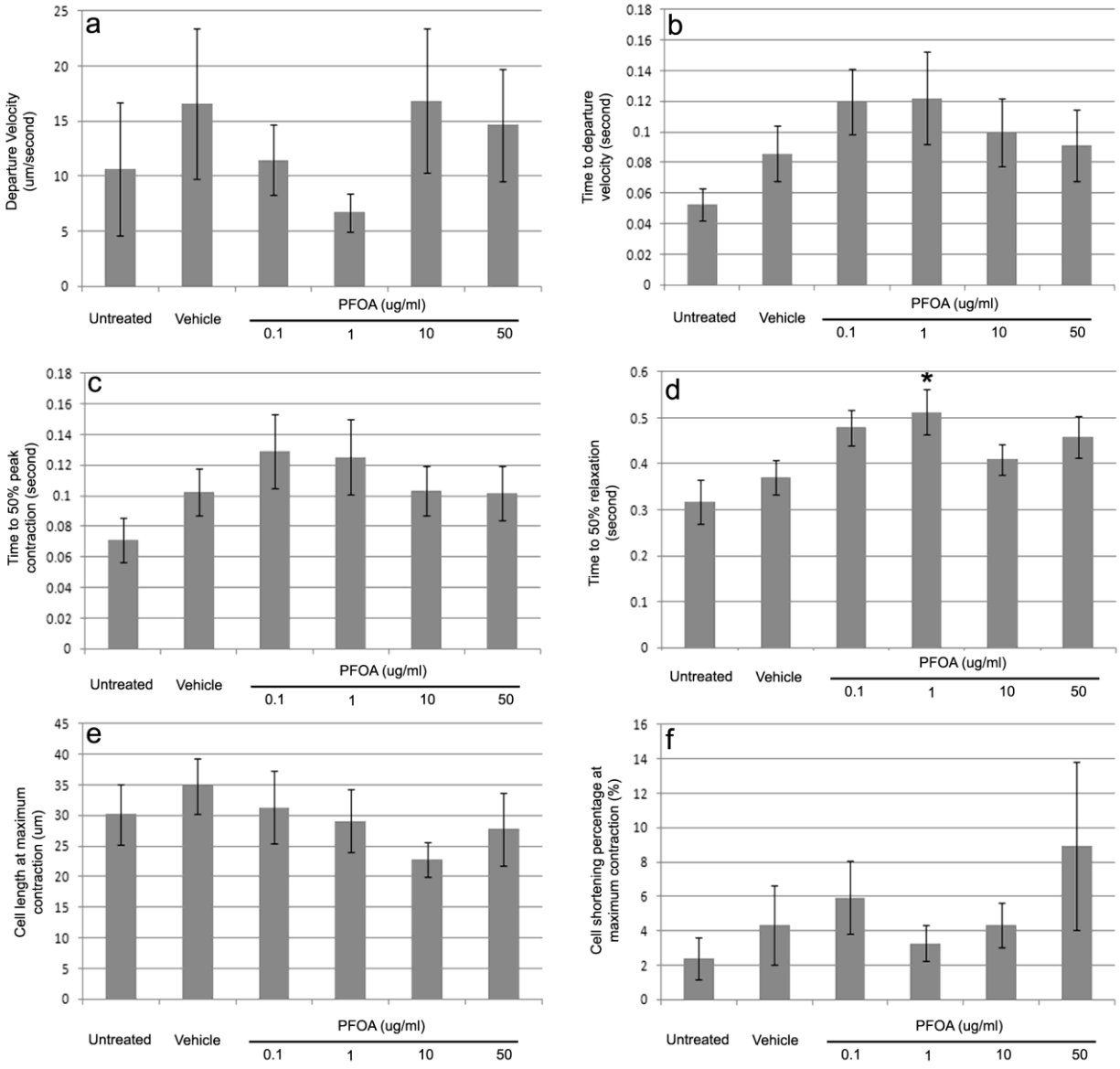

Figure 4.5 Contractility of primary cardiomyocytes exposed to PFOA in vitro. Unexposed fertile chicken eggs were incubated until embryonic day ten and primary cardiomyocytes were isolated from embryos. Three independent isolations of primary cardiomyocytes were used. Following 24 hours of incubation, cardiomyocytes were exposed to 0, 0.1, 1, 10 or 50 µg/ml PFOA in 0.1%

DMSO and incubated for one hour. (a) Departure velocity (N=9-16). (b) Time to departure velocity (N=9-16). (c) Time to 50% peak contraction (N=9-16). (d) Time to 50% relaxation (N=9-16). (e) Cell length at maximum contraction (N=9-16). (f) Cell shortening percentage at maximum contraction (N=9-16). Data are presented as mean ± SEM. * Denotes statistical difference from vehicle group ($P<0.05$).

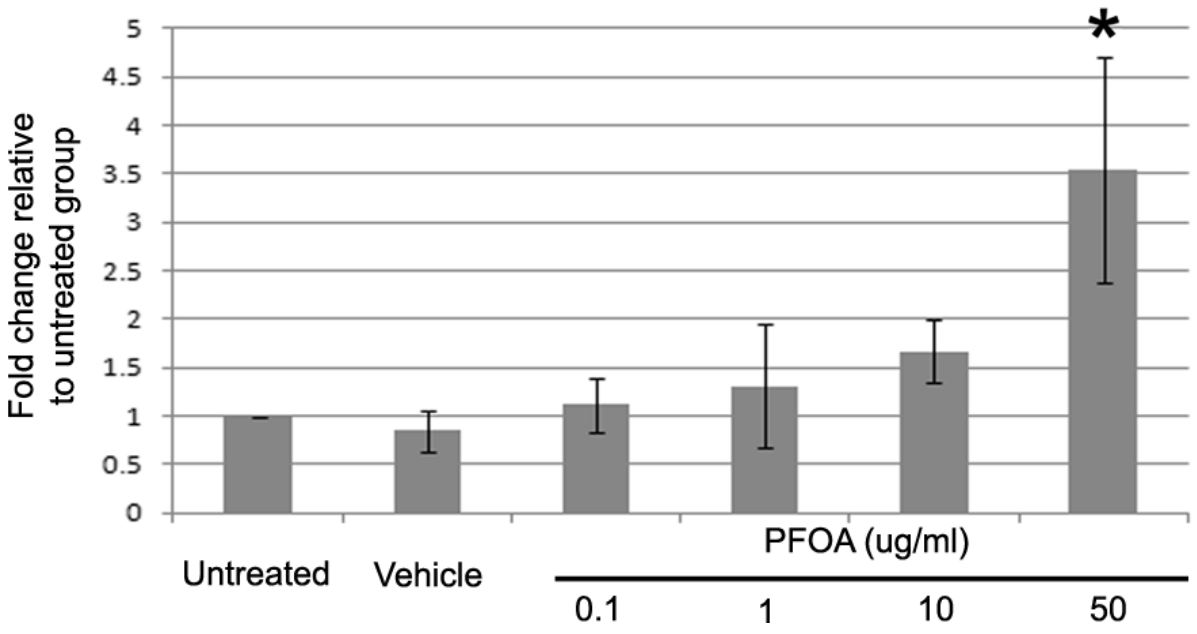

Figure 4.6 Reactive oxygen species (ROS) generation in primary cardiomyocytes exposed to PFOA in vitro. Unexposed fertile chicken eggs were incubated until embryonic day ten and primary cardiomyocytes were isolated from embryos. Following 24 hours of incubation, cardiomyocytes were incubated with 20 µM 2',7'-dichlorofluorescein (DCF) for 30 minutes and then incubated with 0, 0.1, 1, 10, or 50 µg/ml PFOA in 0.1% DMSO for one hour. Fold change relative to the vehicle group was assessed (N=3). Data are presented as mean ± SD. * Denotes statistical difference from vehicle group ($P<0.05$).

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

During this dissertation work, developmental cardiotoxicity induced by PFOA in a chicken embryo model was identified and studied. The detailed results are reported in three manuscripts (Chapters 3-5). The first manuscript (Chapter 3) focused on the morphological and functional changes induced by PFOA in hearts of late stage chicken embryos and hatchling chickens and was published in *Toxicology*; the second manuscript (Chapter 4) focused on molecular mechanisms of PFOA-induced developmental cardiotoxicity and has been accepted for publication in the *Journal of Toxicology and Environmental Health Part A*; the third manuscript (Chapter 5) focused on cellular mechanisms of PFOA-induced developmental cardiotoxicity and will be submitted to the *Archives of Environmental Contamination and Toxicology*.

In the studies that were summarized in my first manuscript, PFOA-induced developmental cardiotoxicity was first identified with a novel histological endpoint: right ventricular wall thickness and the thickness of a dense layer of myosin staining within the right ventricular wall. This histological endpoint was then confirmed with echocardiography, which measured associated morphological and functional alterations. These endpoints indicated that in ovo PFOA exposure altered heart development and that the left ventricle behaved like an early hypertensive heart in the compensation stage. This was a completely new endpoint identified for an organism exposed to PFOA.

In my second manuscript, the mechanisms of toxicity for PFOA-induced developmental cardiotoxicity were assessed. WY 14,643, a known agonist of PPARα, failed to mimic

histological changes after in ovo exposure as no difference in the thickness of the right ventricular wall or in the thickness of the dense layer of myosin staining were observed. In ovo WY 14,643 exposure did, however, mimic most of the morphological and functional changes to the left ventricle induced by PFOA when assessed with echocardiography, suggesting partial involvement of PPARα. Pathways alternative to PFOA agonism were then investigated, including inflammatory cytokines and the BMP2 signaling pathway. Following PFOA exposure, alterations of BMP2, its second messenger, and downstream target gene expression levels were observed in early embryos exposed to PFOA in ovo. These changes suggest that the mechanisms of PFOA-induced developmental cardiotoxicity are a combination of PPARα activation and interference with the BMP2-SMAD-Nkx2.5/GATA4 pathway.

In the third manuscript, the mechanisms of PFOA-induced cardiotoxicity were further investigated with primary cardiomyocyte culture model. Primary cardiomyocytes were exposed to PFOA via two methods: in vitro (cells were isolated from hearts of unexposed ED10 embryos and were then exposed to PFOA in medium) and pre-exposed (cells were isolated from hearts of ED10 embryos exposed to PFOA in ovo, an exposure scenario identical to the in vivo tests). When viability of cells exposed to PFOA in vitro was assessed, direct exposure of cells to PFOA induced cytotoxicity only at high doses (75 and 100 µg/ml). When viability of cells pre-exposed to PFOA in ovo was assessed under a challenge (serum starvation), no cytotoxicity was observed. When the contractility of individual cardiomyocytes was examined, cells from hearts of pre-exposed embryos had similar morphological and functional changes as hearts from the exposed hatchling chickens. Individual cardiomyocytes from cells that were exposed in vitro only had a slower relaxation time, which was somewhat inconsistent with observations made on hearts of exposed hatchling chickens. Additionally, ROS were only significantly induced

following a relatively high concentration of PFOA (50 μg/ml) in medium. These data suggest that PFOA induces cardiotoxicity on early upstream signaling pathways in heart development and is likely independent of cytotoxicity in cardiomyocytes from direct PFOA contact.

In summary, the work described in the three chapters together demonstrates that PFOA induces developmental cardiotoxicity in an avian model. When developing hearts of chicken embryos are exposed to PFOA, cardiotoxicity occurs in the form of morphological and functional changes. WY 14,643 experiments indicated that PPARα is involved, but because of differences observed in cardiac effects between PFOA-exposed and WY 14,643-exposed embryos, PPARα signaling is not the only contributing factor. Molecular studies with early stage embryos identified the BMP2-SMAD-Nkx2.5/GATA4 pathway as another contributing factor. At the cellular level, while PFOA is capable of inducing overt cytotoxicity and ROS generation at high concentrations when applied directly to cardiomyocytes, the interference of PFOA in early signaling pathways seems to be responsible for the developmental cardiotoxicity observed in vivo, as confirmed with directly-exposed and in ovo exposed primary cardiomyocytes.

My dissertation work demonstrated that PFOA exposure leads to developmental cardiotoxicity in an avian model. The doses used in my studies are higher than exposure levels in the general human population: 0.5 mg of PFOA/kg of egg weight, the lowest dose used in the in vivo studies, resulted in a serum concentration of 1230.8 ng/ml. The median serum concentration of PFOA in the general human population between 2009 and 2010 was 3.2 ng/ml (CDC 2013). The relatively large margin of exposure indicates that the developmental cardiotoxicity we observed is not likely an immediate threat to public health. However, in relatively highly exposed human populations, exposure levels are much higher and closer to the doses in our studies. The high values from serum concentration data identified by epidemiological studies

(5,100 ng/ml in Olsen et al. 2007 and 17,556.6 ng/ml in Steenland et al. 2009) exceeded the values resulting from lower doses administered in our studies. However, no published data are currently available on the cardiac health of the offspring of highly-exposed humans. More studies are necessary to determine whether the cardiac health of offspring of highly-exposed humans is negatively impacted.

While nominal immediate human health risks are associated with this particular study, my dissertation work revealed the potential of developmental cardiotoxicity following exposure to an environmental contaminant and united several novel methods for evaluating effects of an environmental agent on heart development. These novel methods included evaluation of morphology and function in living organisms with echocardiography, histological assessment of post-mortem tissues, assessment of morphology and function at the cellular level, and molecular biological techniques to evaluate molecular and genetic signaling pathways. My work could facilitate the establishment of developmental cardiotoxicity screening test standards.

5.2 Pitfalls and potential improvements

In this dissertation work, I identified a novel endpoint of PFOA-induced development toxicity and evaluated mechanisms of toxicity. I associated PFOA-induced developmental cardiotoxicity with the PPARα pathway and the BMP2-SMAD-Nkx2.5/GATA4 pathway and found that early stage interference with cardiomyocyte development rather than overt cytotoxicity is likely a mechanism at the cellular level. However, there were some potential drawbacks of these experiments and if I had the chance, I would have made a few modifications to my experiments.

- First, as the functional parameters measured by echocardiography in hatchling chickens developmentally exposed to PFOA had biphasic responses, additional PFOA doses may have helped me to better understand the dose-response. The histological evaluation focused on the right ventricle while echocardiography assessments focused on the left ventricle; therefore, the relationship between the morphological changes in left and right ventricles remains unclear. The big picture of the morphological and functional changes could be clearer had I been able to assess the left ventricle histologically and the right ventricle with echocardiography.
- The mechanistic studies on PPARα relied on the selective PPARα agonist WY 14,643. While this chemical is generally accepted as a selective PPARα agonist, inclusion of an antagonist such as GW6471 to block the PPARα pathway, would have further strengthen my conclusions. Similarly, if RNAi gene silencing could have been used in the primary cardiomyocyte cultures to knock down PPARα, this would have also strengthened my conclusions regarding PPARα signaling. My data suggest that there are interactions between the PPARα and the BMP2 signaling pathways, but not all of the experiments included both PFOA and WY 14,643 to compare the responses. This limited my conclusions about the potential interactions between PPARα and BMP2 signaling. Additionally, the mechanistic work based in early chicken embryos could have better demonstrated the gene expression changes if I had worked with earlier stage isolated hearts rather than embryonic day four embryos and day sixe hearts, especially for genes expressed very early in development such as Nkx2.5 and GATA4. The dose-dependent differences in mRNA levels might have been more evident in ED4 or earlier stage hearts.

5.3 Future directions

PFOA is an important industrial and consumer compound that has become a widespread environmental contaminant. It has been intensively studied for its potential toxicity but many effects remain unknown. In this dissertation, developmental cardiotoxicity was identified in hearts of chicken embryos and hatchlings following PFOA exposure. Mechanistic work revealed some of the mechanisms of toxicity. However, much still remains to be done in regard to the effects of PFOA and related compounds on heart development.

First, the long-term health effects of the developmental cardiotoxicity need to be determined. Instead of terminating animals within 24 hours of hatching, animals should be kept until they reach adulthood. Assessment of morphological and functional parameters should be done to determine the persistence of the developmental cardiotoxicity. Discerning whether the effects resolve or persist will have important implications for human and environmental health.

Second, alternative mechanisms should still be explored. Current work indicated that agonsim of PPARα and interference with the BMP2 signaling pathway were potential mechanisms of the cardiotoxicity, but additional mechanisms likely exist. First, it is necessary to assess additional inflammatory cytokines. mRNA expressions of the cytokines measured in this work did not significantly differ. However, only three cytokines were measured and they were measured at one specific time point. Embryonic development involves many rapidly changing signals; it is possible that these, and other inflammatory cytokines influence the observed effects, but at different points in development. Furthermore, it is possible that the cardiotoxicity arose from mechanisms that did not directly impact the heart. For example, if PFOA disrupts vasculature resistance or alters the sensitivity of the sympathetic nervous system, elevated

circulation pressure could contribute to the effects that were observed in vivo. Additional work is necessary to evaluate whether PFOA alters these factors to have an impact on the heart.

Another potential research direction is the interaction between PFOA and other developmental cardiotoxicants, such as 2,3,7,8-tetrachlorodibenzodioxin and polychlorinated biphenyls. In the real world, PFOA exposure does not occur by itself and it is capable of inducing synergistic effects with other toxicants (Rodea-Palomares et al. 2011). When combined with other developmental cardiotoxicants, the toxicity could be additive or multiplicative and increase the risk. Additional work on the combined effects of PFOA and other toxicants improve understanding on the combined real risk of exposure to environmental contaminants and help improve human health.

In addition to PFOA, other related perfluorinated compounds such as PFOS, PFHxS, and even drugs that act through PPARs, such as fibrates and thiazolidinediones, could potentially have similar effects on heart development due to structural or mechanistic similarities. Examining these compounds to assess the potential disruption of heart development will provide additional insights into the potential mechanisms of toxicity.

Developmental cardiotoxicity is a relatively understudied area of toxicological research. However, the potential risk that it poses to human health should not be underestimated. Congenital heart diseases are not the only outcome of developmental cardiotoxicity. My speculation is that cardiovascular diseases in adulthood are partially caused by developmental cardiotoxicity before birth and/or in early postnatal stages.

With more work, my ultimate goal regarding developmental cardiotoxicity is to establish a universal test set that could be used to screen for potential developmental cardiotoxicity of

known and unknown compounds. This could help improve human health by decreasing both the rate of congenital heart disease and adult cardiovascular diseases.

REFERENCES

Abbott, B.D., Wood, C.R., Watkins, A.M., Tatum-Gibbs, K., Das, K.P. and Lau, C. (2012). Effects of perfluorooctanoic acid (PFOA) on expression of peroxisome proliferator-activated receptors (PPAR) and nuclear receptor-regulated genes in fetal and postnatal CD-1 mouse tissues. *Reprod. Toxicol.* **33**, 491–505.

Apelberg, B.J., Goldman, L.R., Calafat, A.M., Herbstman, J.B., Kuklenyik, Z., Heidler, J., Needham, L.L., Halden, R.U. and Witter, F.R. (2007). Determinants of fetal exposure to polyfluoroalkyl compounds in Baltimore, Maryland. *Environ. Sci. Technol.* **11**, 3891–3897.

Backes, J.M., Gibson, C.A., Ruisinger, J.F. and Moriarty, P.M. (2007). Fibrates: what have we learned in the past 40 years? *Pharmacotherapy* **27**, 412–424.

Baranowski, M., Blachnio-Zabielska, A. and Gorski, J. (2009).Peroxisome proliferator-activated receptor α activation induces unfavourable changes in fatty acid composition of myocardial phospholipids. *J. Physiol. Pharmacol.* **60**, 13–20.

Barron, M., Gao, M. and Lough, J. (2000). Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. *Dev. Dyn.* **218**, 383–393.

Barth, J.L., Clark, C.D., Fresco, V.M., Knoll, E.P., Lee, B., Argraves, W.S. and Lee, K.H. (2010). Jarid2 is among a set of genes differentially regulated by Nkx2.5 during outflow tract morphogenesis. *Dev. Dyn.* **239**, 2024–2033.

Beaufort-Krol, G.C., Schasfoort-van Leeuwen, M.J., Stienstra, Y. and Bink-Boelkens, M.T. (2007). Longitudinal echocardiographic follow-up in children with congenital complete atrioventricular block. Pacing. *Clin. Electrophysiol.* **30**, 1339–1343.

Berthiaume, J.M. and Wallace, K.B. (2007). Adriamycin-induced oxidative mitochondrial cardiotoxicity. *Cell Biol. Toxicol.* **23**, 15-25.

Bézie, Y., Mesnard, L., Longrois, D., Samson, F., Perret, C., Mercadier, J.J. and Laurent, S. (1996). Interactions between endothelin-1 and atrial natriuretic peptide influence cultured chick cardiac myocyte contractility. *Eur J Pharmacol* **311**, 241–248.

Biegel, L.B., Hurtt, M.E., Frame, S.R., O'Connor, J.C. and Cook, J.C. (2001). Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. *Toxicol. Sci.* **60**, 44–55.

Black, B.L. and Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell. Dev. Biol.* **14**, 167–196.

Bossi, R., Riget, F.F., Dietz, R., Sonne, C., Fauser, P., Dam, M. and Vorkamp, K. (2005). Preliminary screening of perfluorooctane sulfonate (PFOS) and other fluorochemicals in fish, birds and marine mammals from Greenland and the Faroe Islands. *Environ. Pollut.* **136**, 323–329.

Buchhorn, R., Hulpke-Wette, M., Ruschewski, W., Ross, R.D., Fielitz, J., Pregla, R., Hetzer, R. and Regitz-Zagrosek, V. (2003). Effects of therapeutic β blockade on myocardial function and cardiac remodelling in congenital cardiac disease. *Cardiol. Young* **13**, 36–43.

Calvé, A., Noiles, W., Sebag, I.A. and Chalifour, L.E. (2012). The impact of doxorubicin and dexrazoxane injection of prepubertal female rats on pregnancy outcome and cardiac function postpartum. *Can. J. Physiol. Pharmacol.* **90**, 1527–1534.

Candau, R., Iorga, B., Travers, F., Barman, T. and Lionne, C. (2003). At physiological temperatures the ATPase rates of shortening soleus and psoas myofibrils are similar. *Biophys. J.* **85**, 3132–3141.

Cappelli, V., Moggio, R., Monti, E., Paracchini, L., Piccinini, F. and Reggiani, C. (1989). Reduction of myofibrillar ATPase activity and isomyosin shift in delayed doxorubicin cardiotoxicity. *J. Mol. Cell. Cardiol*. **21**, 93–101.

CDC (Centers for Disease Control and Prevention). (2011). National Report on Human Exposure to Environmental Chemicals. Serum Perfluorooctanoic acid (PFOA). http://www.cdc.gov/exposurereport/data_tables/LBXPFOA_DataTables.html [Accessed August 03 2011]

CDC (Centers for Disease Control and Prevention). (2012). Fourth National Exposure Report, Updated Tables, February 2012. http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Feb2012.pdf [Accessed May 30 2012]

CDC (Centers for Disease Control and Prevention). (2013). Fourth National Exposure Report, Updated Tables, September 2012. http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Sep2012.pdf [Accessed Jan 16 2013]

Chang, A.N., Harada, K., Ackerman, M.J. and Potter, J.D. (2005). Functional consequences of hypertrophic and dilated cardiomyopathy-causing mutations in α-tropomyosin. *J. Biol. Chem.* **280**, 34343–34349.

Charon, P. (2004). Etienne Geoffroy Saint-Hilaire (1772-1844) and anencephaly: Contribution of one naturalist to medical knowledge. *Hist. Sci. Med.* **38**, 365-383.

Charron, F. and Nemer, M. (1999). GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10**, 85–91.

Chen, D., Zhao, M. and Mundy, G.R. (2004). Bone Morphogenetic Proteins. *Growth Factors* **22**, 233–241.

DeWitt, J.C., Copeland, C.B., Strynar, M.J. and Luebke, R.W. (2008). Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. *Environ. Health Perspect.* **116**, 644–50.

DeWitt, J.C., Copeland, C.B. and Luebke, R.W. (2009). Suppression of humoral immunity by perfluorooctanoic acid is independent of elevated serum corticosterone concentration in mice. *Toxicol. Sci.* **109**, 106–112.

DeWitt, J.C., Shnyra, A., Badr, M.Z., Loveless, S.E., Hoban, D., Frame, S.R., Cunard, R., Anderson, S.E., Meade, B.J., Peden-Adams, M.M., Luebke, R.W. and Luster, M.I. (2009). Immunotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate and the role of peroxisome proliferator-activated receptor α. *Crit. Rev. Toxicol.* **39**, 76–94.

Djouadi, F., Brandt, J.M., Weinheimer, C.J., Leone, T.C., Gonzalez, F.J. and Kelly, D.P. (1999). The role of the peroxisome proliferator-activated receptor α (PPARα) in the control of cardiac lipid metabolism. *Prostaglandins Leukot. Essent. Fatty Acids* **60**, 339–43.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992). Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68**, 879–887.

Dukanović, N., Jakovljević, V. and Mujović, V.M. (2009). Evaluation of myocardial relaxation in conditions of cardiac remodeling. *Med. Pregl.* **62**, 555–568.

DuPont. (2008). Information on PFOA. http://www2.dupont.com/PFOA2/en_US/index.html [Accessed August 03 2011]

Emmett, E.A., Shofer, F.S., Zhang, H., Freeman, D., Desai, C. and Shaw, L.M. (2006). Community exposure to perfluorooctanoate: relationships between serum concentrations and exposure sources. *J. Occup. Environ. Med.* **48**, 759–770.

Eriksen, K.T., Raaschou-Nielsen, O., Sørensen, M., Roursgaard, M., Loft, S. and Møller, P. (2010). Genotoxic potential of the perfluorinated chemicals PFOA, PFOS, PFBS, PFNA and PFHxA in human HepG2 cells. *Mutat. Res.* **700**, 39–43.

Eriksson, J.G. (2006). Early growth, and coronary heart disease and type 2 diabetes: experiences from the Helsinki Birth Cohort Studies. *Int. J. Obes. (Lond.)* **30 Supply 4**, S18–22.

Falandysz, J., Taniyasu, S., Yamashita, N., Rostkowski, P., Zalewski, K. and Kannan, K. (2007). Perfluorinated compounds in some terrestrial and aquatic wildlife species from Poland. *J. Environ. Sci. Health A*. **42**, 715–719.

Farrell, M.J. and Kirby, M.L. (2001). Cell Biology of Cardiac Development. *Int. Rev. Cytol.* **202**, 99–158.

Feige, J.N., Gelman, L., Michalik, L., Desvergne, B. and Wahli, W. (2006). From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog. Lipid Res.* **45**, 120–159.

Fiedlera, S., Pfistera, G. and Schramma, K. (2010). Poly- and perfluorinated compounds in household consumer products. *Toxicol. Environ. Chem.* **92**, 1801–1811.

Finck, B.N., Lehman, J.J., Leone, T.C., Welch, M.J., Bennett, M.J., Kovacs, A., Han, X., Gross, R.W., Kozak, R., Lopaschuk, G.D. and Kelly, D.P. (2002). The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. *J. Clin. Invest.* **109**, 121–130.

Frisbee, S.J., Shankar, A., Knox, S.S., Steenland, K., Savitz, D.A., Fletcher, T. and Ducatman, A.M. (2010). Perfluorooctanoic acid, perfluorooctanesulfonate, and serum lipids in children and adolescents: results from the C8 Health Project. *Arch. Pediatr. Adolesc. Med.* **164**, 860–69.

Frömel, T. and Knepper, T.P. (2010). Fluorotelomer ethoxylates: sources of highly fluorinated environmental contaminants part I: biotransformation. *Chemosphere.* **80**, 1387–1392.

Gallo, V., Leonardi, G., Genser, B., Lopez-Espinosa, M.J., Frisbee, S.J., Karlsson, L., Ducatman, A.M. and Fletcher, T. (2012). Serum perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) concentrations and liver function biomarkers in a population with elevated PFOA exposure. *Environ. Health Perspect.* **120**, 655–660.

Go, A.S., Mozaffarian, D., Roger, V.L., Benjamin, E.J., Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., Franco, S., Fullerton, H.J., Gillespie, C., Hailpern, S.M., Heit, J.A., Howard, V.J., Huffman, M.D., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Magid, D., Marcus, G.M., Marelli, A., Matchar, D.B., McGuire, D.K., Mohler, E.R., Moy, C.S., Mussolino, M.E., Nichol, G., Paynter, N.P., Schreiner, P.J., Sorlie, P.D., Stein, J., Turan, T.N., Virani, S.S., Wong, N.D., Woo, D., Turner, M.B., on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. 2013. *Circulation* **127**, 143-152.

Grandjean, P., Andersen, E.W., Budtz-Jørgensen, E., Nielsen, F., Mølbak, K., Weihe, P. and Heilmann, C. (2012). Serum vaccine antibody concentrations in children exposed to perfluorinated compounds. *JAMA* **307**, 391–397.

Grimes, A.C., Erwin, K.N., Stadt, H.A., Hunter, G.L., Gefroh, H.A., Tsai, H.J. and Kirby, M.L. (2008). PCB126 exposure disrupts zebrafish ventricular and branchial but not early neural crest development. *Toxicol. Sci.* **106**, 193–205.

Hantson, P. and Beauloye, C. (2012). Myocardial metabolism in toxin-induced heart failure and therapeutic implications. *Clin. Toxicol. (Phila).* **50**, 166-171.

Harvey, R.P., Lai, D., Elliott, D., Biben, C., Solloway, M., Prall, O., Stennard, F., Schindeler, A., Groves, N., Lavulo, L., Hyun, C., Yeoh, T., Costa, M., Furtado, M. and Kirk, E. (2002). Homeodomain factor Nkx2-5 in heart development and disease. *Cold Spring Harb. Symp. Quant. Biol.* **67**, 107–114.

Henshel, D.S., DeWitt, J. and Troutman, A. (2003). Using chicken embryos for teratology studies. *Curr. Protoc. Toxicol.* **Chapter 13**: Unit 13.4.1–19.

Hoffman, K., Webster, T.F., Bartell, S.M., Weisskopf, M.G., Fletcher, T. and Vieira, V.M. (2011). Private drinking water wells as a source of exposure to perfluorooctanoic acid (PFOA) in communities surrounding a fluoropolymer production facility. *Environ. Health Perspect.* **119**, 92–97.

Ibarra-Lara, L., Hong, E., Soria-Castro, E., Torres-Narváez, J.C., Pérez-Severiano, F.,Del Valle-Mondragón, L., Cervantes-Pérez, L.G., Ramírez-Ortega, M., Pastelín-Hernández, G.S. and Sánchez-Mendoza, A.(2012). Clofibrate PPARα activation reduces oxidative stress and improves ultrastructure and ventricular hemodynamics in no-flow myocardial ischemia. *J. Cardiovasc. Pharmacol.* **60**, 323–334.

Jiang, Q., Lust, R.M., Strynar, M.J., Dagnino, S. and DeWitt, J.C. (2012). Perflurooctanoic acid induces developmental cardiotoxicity in chicken embryos and hatchlings. *Toxicology* **293**, 97–106.

Jones, S.P. and Kennedy, S.W. (2009). Chicken embryo cardiomyocyte cultures--a new approach for studying effects of halogenated aromatic hydrocarbons in the avian heart. *Toxicol Sci.* **109**, 66–74.

Juang, J.M., de Las Fuentes, L., Waggoner, A.D., Gu, C.C. and Dávila-Román, V.G. (2010). Association and interaction of PPAR-complex gene variants with latent traits of left ventricular diastolic function. *BMC Med. Genet.* **11**, 65.

Kannan, K., Corsolini, S., Falandysz, J., Fillmann, G., Kumar, K.S., Loganathan, B.G., Mohd, M.A., Olivero, J., Van Wouwe, N., Yang, J.H. and Aldoust, K.M. (2004). Perfluorooctanesulfonate and related fluorochemicals in human blood from several countries. *Environ. Sci. Technol.* **38**, 4489–4495.

Karam, W.G. and Ghanayem, B.I. (1997). Induction of replicative DNA synthesis and PPARα-dependent gene transcription by Wy-14 643 in primary rat hepatocyte and non-parenchymal cell co-cultures. *Carcinogenesis* **18**, 2077–2083.

Kettunen, P., Nie, X., Kvinnsland, I.H. and Luukko, K. (2006). Histological development and dynamic expression of Bmp2-6 mRNAs in the embryonic and postnatal mouse cranial base. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **288**, 1250–1258.

Kirby, M.L. (2002). Molecular embryogenesis of the heart. *Pediatr. Dev. Pathol.* **5**, 516–543.

Knox, S.S., Jackson, T., Frisbee, S.J., Javins, B. and Ducatman, A.M. (2011). Perfluorocarbon exposure, gender and thyroid function in the C8 Health Project. *J. Toxicol. Sci.* **36**, 403–410.

Kobayashi, S., Lackey, T., Huang, Y., Bisping, E., Pu, W.T., Boxer, L.M. and Liang, Q. (2006). Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. *FASEB J.* **20**, 800–802.

Koren, M.J., Devereux, R.B., Casale, P.N., Savage, D.D. and Laragh, J.H. (1991). Relation of left ventricular mass and geometry to morbidity and mortality in uncomplicated essential hypertension. *Ann. Intern. Med.* **114**, 345–352.

Kuo, P.L., Lee, H., Bray, M.A., Geisse, N.A., Huang, Y.T., Adams, W.J., Sheehy, S.P. and Parker, K.K. (2012). Myocyte shape regulates lateral registry of sarcomeres and contractility. *Am. J. Pathol.* **181**, 2030–2037.

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C. and Leiden, J.M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes. Dev.* **11**, 1048–1060.

Ladd, A.N., Yatskievych, T.A. and Antin, P.B. (1998). Regulation of avian cardiac myogenesis by activin/TGFβ and bone morphogenetic proteins. *Dev. Biol.* **204**, 407–419.

Langley-Evans, S.C. (2006). Developmental programming of health and disease. *Proc. Nutr. Soc.* **65**, 97–105.

Lau, C., Butenhoff, J.L. and Rogers, J.M. (2004). The developmental toxicity of perfluoroalkyl acids and their derivatives. *Toxicol. Appl. Pharmacol.* **198**, 231–241.

Li, C., Li, J., Cai, X., Sun, H., Jiao, J., Bai, T., Zhou, X.W., Chen, X., Gill, D.L. and Tang, X.D. (2011). Protein kinase D3 is a pivotal activator of pathological cardiac hypertrophy by selectively increasing the expression of hypertrophic transcription factors. *J. Biol. Chem.* **286**, 40782–40791.

Lionne, C., Iorga, B., Candau, R. and Travers, F. (2003). Why choose myofibrils to study muscle myosin ATPase? *J. Muscle. Res. Cell. Motil.* **24**, 139–148.

Liu, C.J., Cheng, Y.C., Lee, K.W., Hsu, H.H., Chu, C.H., Tsai, F.J., Tsai, C.H., Chu, C.Y., Liu, J.Y., Kuo, W.W. and Huang, C.Y. (2008). Lipopolysaccharide induces cellular hypertrophy through calcineurin/NFAT-3 signaling pathway in H9c2 myocardiac cells. *Mol. Cell. Biochem.* **313**, 167–178.

Luo, D.D., Fielding, C., Phillips, A. and Fraser, D. (2009). Interleukin-1 β regulates proximal tubular cell transforming growth factor β-1 signalling, *Nephrol. Dial. Transplant.* **24**, 2655–2665.

Lutin, W.A., Brumund, M.R., Jones, C., Tharpe, C.E., Montegomery, M. and McCaffrey, F.M. (1999). Hemodynamic abnormalities in fetuses with congenital heart disease. *Pediatr. Cardiol.* **20**, 390–395.

Maloney, E.K. and Waxman, D.J. (1999). trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicol. Appl. Pharmacol.* **161**, 209–218.

Martin, J.W., Whittle, D.M., Muir, D.C. and Mabury, S.A. (2004). Perfluoroalkyl contaminants in a food web from Lake Ontario. *Environ. Sci. Technol.* **38**, 5379–5385.

McCulley, D.J. and Black, B.L. (2012). Transcription Factor Pathways and Congenital Heart Disease. *Curr. Top Dev. Biol.* **100**, 253–77.

Meade, K.G., Higgs, R., Lloyd, A.T., Giles, S. and O'Farrelly, C. (2009). Differential antimicrobial peptide gene expression patterns during early chicken embryological development. *Dev. Comp. Immunol.* **33**, 516–524.

Montanez, J.E., Peters, J.M., Correll, J.B., Gonzalez, F.J. and Patterson, A.D. (2013). Metabolomics: An Essential Tool to Understand the Function of Peroxisome Proliferator-Activated Receptor A. *Toxicol. Pathol.* **41**, 410-418.

Nolan, L.A., Nolan, J.M., Shofer, F.S., Rodway, N.V. and Emmett, E.A. (2009). The relationship between birth weight, gestational age, and perfluorooctanoic acid (PFOA)-contaminated public drinking water. *Reprod. Toxicol.* **27**, 231–238.

Nolan, L.A., Nolan, J.M., Shofer, F.S., Rodway, N.V. and Emmett, E.A. (2010). Congenital anomalies, labor/delivery complications, maternal risk factors and their relationship with perfluorooctanoic acid (PFOA)-contaminated public drinking water. *Reprod. Toxicol.* **29**, 147–155.

O'Brien, J.M., Crump, D., Mundy, L.J., Chu, S., McLaren, K.K., Vongphachan, V., Letcher, R.J. and Kennedy, S.W. (2009). Pipping success and liver mRNA expression in chicken embryos exposed in ovo to C8 and C11 perfluorinated carboxylic acids and C10 perfluorinated sulfonate. *Toxicol. Lett.* **190**, 134–139.

Olivotto, I., Girolami, F., Nistri, S., Rossi, A., Rega, L., Garbini, F., Grifoni, C., Cecchi, F. and Yacoub, M.H. (2009). The many faces of hypertrophic cardiomyopathy: from developmental biology to clinical practice. *J. Cardiovasc. Transl. Res.* **2**, 349–367.

Olsen, G.W., Gilliland, F.D., Burlew, M.M., Burris, J.M., Mandel, J.S. and Mandel, J.H. (1998). An epidemiologic investigation of reproductive hormones in men with occupational exposure to perfluorooctanoic acid. *J. Occup. Environ. Med.* **40**, 614–622.

Olsen, G.W., Church, T.R., Miller, J.P., Burris, J.M., Hansen, K.J., Lundberg, J.K., Armitage, J.B., Herron, R.M., Medhdizadehkashi, Z., Nobiletti, J.B., O'Neill, E.M., Mandel, J.H. and Zobel, L.R. (2003). Perfluorooctanesulfonate and other fluorochemicals in the serum of American Red Cross adult blood donors. *Environ. Health Perspect.* **111**, 1892–1901.

Olsen, G.W., Burris, J.M., Ehresman, D.J., Froehlich, J.W., Seacat, A.M., Butenhoff, J.L. and Zobel, L.R. (2007). Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. *Environ. Health Perspect.* **115**, 1298–1305.

Palkar, P.S., Anderson, C.R., Ferry, C.H., Gonzalez, F.J. and Peters, J.M. (2010). Effect of prenatal peroxisome proliferator-activated receptor α (PPARα) agonism on postnatal development. *Toxicology* **276**, 79–84.

Peters, J.M., Cheung, C. and Gonzalez, F.J. (2005). Peroxisome proliferator-activated receptor-α and liver cancer: where do we stand? *J. Mol. Med.* **83**, 774–785.

Pu, W. T., Ishiwata, T., Juraszek, A. L., Ma, Q. and Izumo, S. (2004). GATA4 is a dosage sensitive regulator of cardiac morphogenesis. *Dev. Biol.* **275**, 235–244.

Recanatini, M., Poluzzi, E., Masetti, M., Cavalli, A. and De Ponti, F. (2005). QT prolongation through hERG K(+) channel blockade: current knowledge and strategies for the early prediction during drug development. *Med. Res. Rev.* **25**, 133–166.

Reiner, J.L., Nakayama, S.F., Delinsky, A.D., Stanko, J.P., Fenton, S.E., Lindstrom, A.B. and Strynar, M.J. (2009). Analysis of PFOA in dosed CD1 mice. Part 1. Methods development for the analysis of tissues and fluids from pregnant and lactating mice and their pups. *Reprod. Toxicol.* **27**, 360–364.

Rodea-Palomares, I., Leganés, F., Rosal, R. and Fernández-Piñas, F. (2011). Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants. *J. Hazard Mater.* **201-202**, 209–218.

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Greenlund, K.J., Hailpern, S.M., Heit, J.A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Makuc, D.M., Marcus, G.M., Marelli, A., Matchar, D.B., McDermott, M.M., Meigs, J.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G., Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-Rosett, J. and American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2011). American Heart Association Statistics Committee and Stroke Statistics Subcommittee Heart disease and stroke statistics—2011 update: a report from the American Heart Association. *Circulation* **123**, e18–e209.

Rosen, M.B., Abbott, B.D., Wolf, D.C., Corton, J.C., Wood, C.R., Schmid, J.E., Das, K.P., Zehr, R.D., Blair, E.T. and Lau, C. (2008). Gene profiling in the livers of wild-type and PPARα-null mice exposed to perfluorooctanoic acid. *Toxicol. Pathol.* **36**, 592–607.

Sambandam, N., Morabito, D., Wagg, C., Finck, B.N., Kelly, D.P. and Lopaschuk, G.D.(2006). Chronic activation of PPARalpha is detrimental to cardiac recovery after ischemia. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H87–H95.

Schultheiss, T.M., Xydas, S. and Lassar, A.B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4203–4214.

Shi, Y., Moon, M., Dawood, S., McManus, B. and Liu P.P. (2011). Mechanisms and management of doxorubicin cardiotoxicity. *Herz.* **36**, 296–305.

Shin, H.M., Vieira, V.M., Ryan, P.B., Steenland, K. and Bartell, S.M. (2011). Retrospective exposure estimation and predicted versus observed serum perfluorooctanoic acid concentrations for participants in the C8 Health Project. *Environ. Health Perspect.* **119**, 1760–1765.

Slotkin, T.A., MacKillop, E.A., Melnick, R.L., Thayer, K.A. and Seidler, F.J. (2008). Developmental neurotoxicity of perfluorinated chemicals modeled in vitro. *Environ. Health Perspect.***116**, 716–722.

Smagulova, F.O., Manuylov, N.L., Leach, L.L. and Tevosian, S.G. (2008). GATA4/FOG2 transcriptional complex regulates Lhx9 gene expression in murine heart development. *BMC Dev. Biol.* **8**, 67.

Steenland, K., Jin, C., MacNeil, J., Lally, C., Ducatman, A., Vieira, V. and Fletcher, T. (2009). Predictors of PFOA levels in a community surrounding a chemical plant. *Environ. Health Perspect.* **117**, 1083–1088.

Steenland, K., Fletcher, T. and Savitz, D.A. (2010). Epidemiologic Evidence on the Health Effects of Perfluorooctanoic Acid (PFOA). *Environ. Health Perspect.* **118**, 1100–1108.

Stein, C.R., Savitz, D.A. and Dougan, M. (2009). Serum levels of perfluorooctanoic acid and perfluorooctane sulfonate and pregnancy outcome. *Am. J. Epidemiol.* **170**, 837–846.

Steinmetz, M., Quentin, T., Poppe, A., Paul, T. and Jux, C. (2005). Changes in expression levels of genes involved in fatty acid metabolism: upregulation of all three members of the PPAR family (α, gamma, delta) and the newly described adiponectin receptor 2, but not adiponectin receptor 1 during neonatal cardiac development of the rat. *Basic Res. Cardiol.* **100**, 263–269.

Stock, M.K. and Metcalfe, J. (1987). Modulation of growth and metabolism of the chick embryo by a brief (72-hr) change in oxygen availability. *J. Exp. Zool.* **1**, S351–S356.

Sturm, S., Bentley, D., Jordan, P., Russell-Yarde, F. and Ruf, T. (2012). No evidence of QT prolongation with supratherapeutic doses of aleglitazar. *J. Cardiovasc. Pharmacol.* **59**, 288–297.

Surai, P.F. (1999). Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. *Br. Poult. Sci.* **40**, 397–405.

Takagi, Y., Bruyere, H.J. Jr. and Nishikawa, T. (1989). Protective effect of ouabain on adriamycin-induced cardiovascular anomalies in chick embryos. *Teratology* **39**, 573–580.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126**, 1269–1280.

Taylor, B.K., Kriedt, C., Nagalingham, S., Dadia, N. and Badr, M. (2005). Central administration of perfluorooctanoic acid inhibits cutaneous inflammation. *Inflamm. Res.* **54**, 235–242.

Torre-Amione, G. (2005). Immune activation in chronic heart failure. *Am. J. Cardiol*. **95**, 3C–8C; 38C–40C.

Tsai, I.C., Chen, M.C., Jan, S.L., Wang, C.C., Fu, Y.C., Lin, P.C. and Lee, T. (2008). Neonatal cardiac multidetector row CT: why and how we do it. *Pediatr. Radiol.* **38**, 438–451.

USEPA (U.S. Environmental Protection Agency). (2011). 2010/2015 PFOA Stewardship Program. http://www.epa.gov/opptintr/pfoa/pubs/stewardship/ [Accessed August 03 2011]

USEPA (U.S. Environmental Protection Agency). (2012). 2010/2015 PFOA Stewardship Program. http://www.epa.gov/opptintr/pfoa/pubs/stewardship/ [Accessed May 30 2012]

USEPA (U.S. Environmental Protection Agency). (2013). 2010/2015 PFOA Stewardship Program, http://www.epa.gov/opptintr/pfoa/pubs/stewardship/ [Accessed Jan 16 2013]

Vaalgamaa, S., V¨ah¨atalo, A.V., Perkola, N. and Huhtala, S. (2011). Photochemical reactivity of perfluorooctanoicacid (PFOA) in conditions representing surface water. *Sci. Total Environ.* **409**, 3043–3048.

Vieira, V.M., Hoffman, K., Shin, H.M., Weinberg, J.M., Webster, T.F. and Fletcher, T. (2013). Perfluorooctanoic Acid exposure and cancer outcomes in a contaminated community: a geographic analysis. *Environ. Health Perspect.* **121**, 318–323.

Walker, M.K., Pollenz, R.S. and Smith, S.M. (1997). Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with 2,3,7,8- tetrachlorodibenzo-p-dioxin-induced heart defects. *Toxicol. Appl. Pharmacol.* **143**, 407–419.

Wang, N., Szostek, B., Buck, R.C., Folsom, P.W., Sulecki, L.M., Capka, V., Berti, W.R. and Gannon, J.T. (2005). Fluorotelomer alcohol biodegradation-direct evidence that perfluorinated carbon chains breakdown. *Environ. Sci. Technol.* **39**, 7516–7528.

Weihe, P., Kato, K., Calafat, A.M., Nielsen, F., Wanigatunga, A.A., Needham, L.L. and Grandjean, P. (2008). Serum concentrations of polyfluoroalkyl compounds in Faroese whale meat consumers. *Environ. Sci. Technol.* **42**, 6291–6295.

Wolf, C.J., Fenton, S.E., Schmid, J.E., Calafat, A.M., Kuklenyik, Z., Bryant, X.A., Thibodeaux, J., Das, K.P., White, S.S., Lau, C.S. and Abbott, B.D. (2007). Developmental toxicity of perfluorooctanoic acid in the CD-1 mouse after cross-foster and restricted gestational exposures. *Toxicol. Sci.* **95**, 462–473.

Woods, C.G., Kosyk, O., Bradford, B.U., Ross, P.K., Burns, A.M., Cunningham, M.L., Qu, P., Ibrahim, J.G. and Rusyn, I. (2007). Time-course investigation of PPARα- and Kupffer cell–dependent effects of WY-14,643 in mouse liver using microarray gene expression. *Toxicol. Appl. Pharmacol.* **225**, 267–277.

Yamane, K., Ihn, H., Asano, Y., Jinnin, M. and Tamaki, K. (2003). Antagonistic effects of TNF-α on TGF-β signaling through down-regulation of TGF-β receptor type II in human dermal fibroblasts. *J. Immunol.* **171**, 3855–3862.

Yang, J.H. (2010). Perfluorooctanoic acid induces peroxisomal fatty acid oxidation and cytokine expression in the liver of male Japanese medaka (Oryzias latipes). *Chemosphere* **81**, 548–552.

Yeh, C.H., Chen, T.P., Lee, C.H., Wu, Y.C., Lin, Y.M. and Lin, P.J. (2006). Cardiomyocytic apoptosis following global cardiac ischemia and reperfusion can be attenuated by peroxisome proliferator-activated receptor α but not gamma activators. *Shock* **26**, 262–270.

Yuan, J., Wu, J., Hang, Z.G., Zhong, X.K., Zhou, L.W. and Yu, B. (2008). Role of peroxisome proliferator-activated receptor α activation in acute myocardial damage induced by isoproterenol in rats. *Chin. Med. J. (Engl).* **121**, 1569–1573.

Zhang, H., Toyofuku, T., Kamei, J. and Hori, M. (2003). GATA-4 regulates cardiac morphogenesis through transactivation of the N-cadherin gene. *Biochem. Biophys. Res. Commun.* **312**, 1033–1038.

Zhu, X. and Lough, J. (1996). Expression of alternatively spliced and cannonical basic fibroblast growth factor mRNAs in the early embryo and developing heart. *Dev. Dyn.* **206**, 139–145.

Zhu, L.Y. and Lin, J.H. (2008). Pollution trend and environmental behavior of perfluorooctanoic acid: a review. *Ying Yong Sheng Tai Xue Bao.* **19**, 1149-1157.

Zungu, M., Felix, R. and Essop, M.F. (2006). Wy-14,643 and fenofibrate inhibit mitochondrial respiration in isolated rat cardiac mitochondria. *Mitochondrion* **6**, 315–322.

APPENDIX A ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

May 15, 2009

Jamie DeWitt, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. DeWitt:

Your Animal Use Protocol entitled, "Developmental Effects of Environmental Toxicants on Chicken Embryos," (AUP #W217) was reviewed by this institution's Animal Care and Use Committee on 5/15/09. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

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Dear Mr. Jiang:

We are in receipt of your request to reproduce your forthcoming article

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