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Cardiac developmental toxicity

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Abstract

Congenital heart disease is a highly prevalent problem with mostly unknown origins. Many cases of CHD likely involve an environmental exposure coupled with genetic susceptibility, but practical and ethical considerations make nongenetic causes of CHD difficult to assess in humans. The development of the heart is highly conserved across all vertebrate species, making animal models an excellent option for screening potential cardiac teratogens. This review will discuss exposures known to cause cardiac defects, stages of heart development that are most sensitive to teratogen exposure, benefits and limitations of animal models of cardiac development, and future considerations for cardiac developmental toxicity research.

Keywords

congenital heart disease; cardiac developmental biology; teratogen exposure; animal models

Introduction

Congenital heart disease (CHD) affects over 1% of live births and is responsible for the vast majority of prenatal losses, but the origins of most CHD are unknown(Hoffman and Kaplan, 2002; Jenkins et al., 2007). The incidence of noninherited causes of CHD is difficult to assess, but Wilson et al. suggest that up to 30% of some types of defects are preventable(Wilson et al., 1998). The prevention of nongenetic cardiac developmental defects has been held back, however, due to a lack of information on the modifiable risk factors for CHD(Jenkins et al., 2007). Limitations in collecting accurate data on maternal or paternal exposures that lead to cardiac developmental defects include parental recall bias and confounding by other factors(Jenkins et al., 2007). These complexities make animal models of cardiac development an important tool for detecting, isolating, and understanding the mechanism of cardiac teratogens. One key to unraveling the mechanism of a cardiac teratogens and applying animal model data to human CHD is a thorough understanding of normal cardiac development. Several model organisms, including mouse, chick, frog, and zebrafish, are commonly used for cardiac developmental biology studies and could be suitable for investigating CHD due to cardiac teratogen exposure(Yelon, 2001). The process of heart development is highly conserved between vertebrate species, but animal models vary in their similarity to the human genetics, anatomy, bioavailability and pharmacokinetic profiles, and routes of exposure; and all of these factors must be considered when choosing an animal model and interpreting results. This review will provide a basic outline of vertebrate cardiac development and the disturbances that can lead to common CHD. The following section discusses known cardiac teratogens. The benefits and limitations of

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different animal models of cardiac development will subsequently be outlined, and finally, the compounds that require further testing for cardiac developmental toxicity will be discussed.

Common congenital heart defects

Heart morphogenesis is a highly conserved process in all vertebrate organisms. Developing vertebrate hearts are nearly indistinguishable from the linear heart tube stage into the early stages of looping and chamber formation(Warkman and Krieg, 2007). Human exposure to cardiac teratogens during the three months before pregnancy and weeks 2 through 7 of gestation are most likely to result in cardiac structural abnormalities, therefore we will focus on the early stages of cardiac development and how irregularities can lead to defects in this section(Jenkins et al., 2007). Chick staging will be used to describe the cardiac developmental events as many of the studies were performed with chick embryos, but Table 1 includes the timeline for early cardiogenesis milestones in human, mouse, chick, frog, and zebrafish.

Heart tube formation

Following implantation into the uterine endometrium, the early embryo is composed of a flat disk of cells called a blastula(Rogers and Kavlock, 1998). Subsequent gastrulation, or reorganization into a trilaminar structure, produces the ectoderm, mesoderm, and endoderm, which go on to form all organs in the body(Rogers and Kavlock, 1998). Cells proliferate and migrate through the primitive streak during gastrulation, setting up the basic morphogenic fields in the embryo(Rogers and Kavlock, 1998). Gastrulation is a period that is very sensitive to teratogen exposure(Rogers and Kavlock, 1998). Organogenesis follows gastrulation, and the heart is the first organ to form in vertebrates(Harvey and Rosenthal, 1999). At HH4, precardiac cells begin migration from the epiblast (the outer layer of a blastula that gives rise to the ectoderm after gastrulation)(Fishman and Chien, 1997). Beginning at approximately HH5, a signal thought to originate from the endoderm induces progenitor cells within the anterior lateral plate mesoderm to commit to a cardiogenic fate and from two crescent-like cardiogenic plates(Fishman and Chien, 1997; Schultheiss et al., 1995). These plates fuse in the midline, forming the primitive heart tube(DeRuiter et al., 1992). The heart tube is composed of an inner layer of endocardium surrounded by myocardium and separated by cardiac jelly, an extracellular matrix(Srivastava, 2001). The tubular heart begins contracting at HH10(Fishman and Chien, 1997). In addition, by stage HH10 the ventricular region of the heart tube begins to loop to the right(Fishman and Chien, 1997). Any disturbances to the cascade of genes necessary for left-right programming can cause abnormal looping, which is characterized as random, anterior or leftward looping(Gittenberger-De Groot et al., 2005; Olson and Srivastava, 1996). This can result in abnormal atrial situs, dextrocardia, or ventricular inversion cardiac defects(Gittenberger-De Groot et al., 2005).

Cardiac Looping

By HH17, the cardiac jelly within the valve forming regions of the heart tube, the outflow tract and the atrioventricular (AV) junction, becomes thicker and begin to develop into swellings of extracellular matrix called cushions, which will eventually differentiate into the atrioventricular and semilunar valves(Fishman and Chien, 1997). The AV cushions also contribute to the interventricular and interatrial septa(Moreno-Rodriguez et al., 1997). The heart finishes the looping period by HH18, and the early heart chambers are in the adult spatial configuration(Moreno-Rodriguez and Krug, 2010). The linear segments of the early heart must be repositioned to properly align the atrial chambers, ventricles, and aortic and pulmonary arteries; and this repositioning involves extensive remodeling of the inner

curvature of the looped heart tube(Srivastava, 2001). Inadequate remodeling of the inner heart curvature will lead to deficient leftward movement of the outflow tract over the atrioventricular canal, which results in a range of outflow tract abnormalities(Gittenberger-De Groot et al., 2005). Double-inlet left ventricle or double-outlet right ventricle are some of the more severe defects that could arise, in less serious cases the result is a ventricular septal defect(Gittenberger-De Groot et al., 2005; Srivastava, 2001).

Cardiac septation and chamber formation

At HH22, the four heart chambers begin to take shape. At this stage of development the primary interatrial septum is formed following fusion with the AV cushions, which closes the ostium primum(McQueen and Ebooks, 2010; Moreno-Rodriguez and Krug, 2010) . When the ostium primum does not fuse properly a primary atrial septum defect will develop. This generally is combined with inadequate fusion of the superior and inferior AV cushions, leading to an atrioventricular septal defect or atrioventricular canal defect(Gittenberger-De Groot et al., 2005). In addition, humans develop a secondary atrial septum, and insufficient formation of the secondary septum leads to atrial septum secundum defects(Gittenberger-De Groot et al., 2005). The septation process is complete by HH30 and the result is four functional heart chambers.

Known cardiac teratogens

Nongenetic causes of CHD include maternal illnesses, therapeutic and nontherapeutic drug exposures, dietary behaviors, and maternal contact with occupational and environmental chemicals. Table 2 lists some known cardiac teratogens. CHD can be difficult to diagnose, and this limits the ability to estimate risk. Severe CHD can be identified due to the immediate effect on cardiac function. Milder CHD can be more difficult detect, especially if there are no immediate physiological consequences. When left untreated, however, these minor cardiac defects can produce severe disorders later in life(Smith, 2010).

A direct correlation between human teratogen exposure and the resulting cardiac defect is the clearest way to establish CHD risk. Practical and ethical considerations, however, make this type of data difficult to collect. Only some states report CHD in a birth defects registry and reporting is usually only for obvious CHD cases with an adverse impact on heart physiology at or shortly after birth(Smith, 2010). Severe cardiovascular dysfunction is incompatible with life for human embryos after approximately 3-4 weeks of gestation, meaning that exposures resulting in early miscarriage are difficult to identify, and studies that quantify CHD at birth will therefore capture only a small percentage of possible outcomes(Smith, 2010). In addition, to identify a change in the incidence of a relatively rare event a large human population must be surveyed. Most of the surveyed population will not have been exposed to the agent under study, meaning that the cases due to that particular agent will represent a very percentage of the population(Smith, 2010). Finally, recall bias is a potential problem because exposures are often identified through parental recall, and the assessment normally takes place after childbirth(Jenkins et al., 2007). Confounding is also a concern, a heart defect attributed to NSAID use, for example, could be confounded by the condition for which the analgesic was taken (i.e. influenza or a febrile illness)(Jenkins et al., 2007).

Because of these difficulties, animal model studies are often necessary to detect and understand cardiac teratogens. Animal models each have benefits and limitations (see Table 3), and differences between humans and animal models potentially include bioavailability and pharmacokinetic profiles, routes of exposure and placental transfer, genetics, and anatomy(Smith, 2010). Despite these differences, animal models have shown promise in identifying and understand the mechanism of cardiac teratogens(Smith, 2010). Other major

factors to consider when choosing an animal model include the availability of reagents, ease of manipulation and storage, and reproducibility, stability, and expense(Ballatori and Villalobos, 2002). Several model organisms, including mouse, chick, frog, and zebrafish, are commonly used for cardiac developmental biology studies and are also suitable for investigating the cardiac developmental toxicology(Yelon, 2001). The features of each animal model are discussed in the next section.

Animal models of cardiac developmental toxicity

Mus musculus *(mouse)*

Mice are often used in developmental and toxicology research, and several features combine to make the mouse one of the most important animal models of congenital heart defects(Moon, 2006; Savolainen et al., 2009). The mouse is a mammal that shares similar embryogenesis and anatomy and physiology with humans; genes, proteins and regulatory programs are largely conserved between human and mouse; and the mouse genome can be manipulated genetically using techniques unavailable in any other model organism(Moon, 2006). Methods for relating cardiac developmental milestones with embryonic age are available(Savolainen et al., 2009).

Disadvantages of the mouse model include high animal husbandry costs, extensive laws and regulations regarding animal use, the relatively small number of embryos available per mouse, and the inability to effectively monitor the embryos. The teratogenic effects of the compound being studied are more difficult to isolate in a mouse model; confounding factors include maternal bioactivation or inactivation of the test compound and lack of placental transfer. Alternate culturing systems that allow for direct exposure to the test compound and improved monitoring capabilities include explant assays and whole embryo culture. Portions of the murine heart can be removed and cultured at various development stages(Kruithof et al., 2003). These explants could be used to determine the effects of a test compound on a specific cardiac morphological event. Whole embryo culture was developed as an *in vitro* screening method for teratogenic chemicals(New, 1978). Early organogenesis-stage rodent embryos (late headfold-early somite stage) can be isolated, and the embryos continue to develop in rotated in culture bottles filled with culture medium for 48-72 hours(Augustine-Rauch et al., 2010). Test compounds are added directly to the culture medium and embryos can be monitored for developmental abnormalities(Augustine-Rauch et al., 2010).

Gallus gallus *(chicken)*

Chicken and quail embryos are a commonly used model system in developmental biology and teratology. Bird and mammal embryos and organs develop in a similar manner, and widely accepted chicken embryo staging methods allow for straightforward classification of developmental age(Ballatori and Villalobos, 2002; Hamburger and Hamilton, 1951; LaBonde, 1991; McMillan, 1990; McQueen and Ebooks, 2010; Moreno-Rodriguez and Krug, 2010). Adult chicken hearts are four-chambered and similar anatomically to mammalian hearts(Moreno-Rodriguez and Krug, 2010). Many avian embryos of the same stage can be obtained at once, and the cost of avian embryos is low when compared with the husbandry costs of mammalian animal model systems(Moreno-Rodriguez and Krug, 2010). The teratogenic effects of the compound being studied are more easily isolated because avian embryos develop outside of the mother. There is no maternal inactivation or bioactivation of test compounds, there is no need to assume that the compound is transferred across the placenta, and changes in concentration and metabolism are more easily determined because the exact time of exposure if known(McQueen and Ebooks, 2010; Mishima et al., 2006; Moreno-Rodriguez and Krug, 2010).

Toxicology experiments with avian embryos can be conducted using several methods that vary in the amount of manipulation that can be performed, the amount of time the embryo can be cultured, and the ease of monitoring. *In ovo* incubation allows a researcher to inject compounds through windowed eggs into the air space or on top of the embryo and continue to culture the embryo within the egg(Darnell and Schoenwolf, 2000; McQueen and Ebooks, 2010; Moreno-Rodriguez and Krug, 2010). The chick can survive to hatching with this method, but monitoring is limited. Shell-less culture involves growing the embryo outside of the shell and on top of the egg yolk, which allows for chick survival until approximately HH36 (day 10) and convenient monitoring(Darnell and Schoenwolf, 2000; McQueen and Ebooks, 2010; Moreno-Rodriguez and Krug, 2010). The compounds being studied can be placed directly onto the embryo or teratogens can be targeted to specific tissues using implants or grafts(Eichele et al., 1984; Tickle et al., 1982). In *ex ovo* culture the whole chick embryo is removed from the egg, stretched over a glass or paper ring, and cultured on an egg-agar substrate or in culture medium(Darnell and Schoenwolf, 2000). Ex ovo cultures can develop from approximately stage HH1 until stage HH14 (day 2), and the *ex ovo* embryos are easily manipulated(Darnell and Schoenwolf, 2000). Finally, tissue explants of regions of interest can be cultured for several days in contact with the compound of interest(Darnell and Schoenwolf, 2000). This technique preserves cell-cell and cell-matrix architecture and relationships and allows for easy monitoring and manipulation. For avian teratology experimentation the culture method must be carefully selected, however, as studies have shown that the culture method itself can cause embryonic abnormalities(Drake et al., 2006). Although transgenic experiments are not possible in chick, in depth genetic investigation is possible with the chick model as there are a range of tools available for investigating molecular mechanisms(Antin and Konieczka, 2005; Brown et al., 2003).

Xenopus *(African clawed frog)*

Xenopus have become a common animal model for studying heart development and also has advantages for studying cardiac developmental toxicology. In general, frog heart development closely resembles higher vertebrate heart development, and embryo staging techniques have been established that match developmental age with cardiac morphological events(Kaltenbrun et al., 2011; Warkman and Krieg, 2007). Many of the same heart development pathways are conserved when compared with mammals, although anatomically the adult frog heart has three chambers, one atria and two ventricles, which differs from the mammalian and avian four-chambered heart(Warkman and Krieg, 2007). A researcher can induce the ovulation of a large number of eggs in Xenopus, and, once fertilized, the eggs provide a large number of embryos at the same developmental stage for experimental studies(Bellerby, 1933; SHAPIRO and ZWARENSTEIN, 1934; Warkman and Krieg, 2007). The cost maintaining a Xenopus colony in the laboratory is low when compared with mammalian husbandry costs(Warkman and Krieg, 2007). The *Xenopus* eggs are relatively large (about 1 mm in diameter) and can be easily manipulated for delivery of foreign RNA, DNA, proteins, or drugs; and the embryos have been shown to heal well after microsurgery(Ballatori and Villalobos, 2002; Warkman and Krieg, 2007). Test compounds can also be added externally to the embryo growth medium. The *Xenopus* embryos can be cultured in a simple salt solution because the egg yolk supports embryonic development, and, due to the embryos' ability to heal after microsurgery, this model works well for tissue explant assays(Kaltenbrun et al., 2011). Xenopus embryos develop externally, meaning that there is no maternal inactivation or bioactivation of test compounds, there is no placental transfer, the exact time of exposure to test compounds is known, and monitoring is possible(Kaltenbrun et al., 2011). In addition, *Xenopus* do not require a functional cardiovascular system for survival during embryogenesis, which allows for the analysis of serious cardiac defects in living embryos(Kaltenbrun et al., 2011). The *Xenopus tropicalis* genome has been sequenced, and molecular biology techniques such as embryonic gene

overexpression or knockdown and transgenesis techniques are well established(Kaltenbrun et al., 2011).

Danio rerio *(zebrafish)*

The zebrafish model was historically used in ecological toxicity testing as a sensitive indicator of water contamination and has since become a useful model for developmental biology, pharmacology, toxicology, and the study of human disease(Augustine-Rauch et al., 2010; Lieschke and Currie, 2007). Cardiac patterning and the genetic regulation of early cardiac morphogenesis in zebrafish mirrors that of higher vertebrates, and embryo staging techniques have been established to standardize the developmental age of cardiac morphological events(Hu et al., 2000; Kimmel et al., 1995; Knaut et al., 2002; Yelon, 2001). The adult zebrafish heart is less anatomically similar to higher vertebrates, however, as it has only two chambers(Lieschke and Currie, 2007). Zebrafish are a cost-effective animal model because they require little equipment, they can produce a large number of progeny, they develop very rapidly (organogenesis is complete by 72 hours postfertilization), and the fish can reach high stocking densities(Augustine-Rauch et al., 2010; Ballatori and Villalobos, 2002; Lieschke and Currie, 2007; Yelon, 2001). The zebrafish embryos are externally fertilized and transparent, which means that the embryos can easily be manipulated and observed (Ballatori and Villalobos, 2002; Lieschke and Currie, 2007). Test compounds can be added directly to the zebrafish aqueous environment to determine their effects; the fish have been found to be permeable to xenobiotics in their surroundings(Ballatori and Villalobos, 2002). Zebrafish development will proceed even in the absence of a functional circulation system, which allows for the analysis of acute defects in living embryo(Pelster and Burggren, 1996; Yelon, 2001). The zebrafish genome has been sequenced and considerable number of molecular biology tools are available for the species(Augustine-Rauch et al., 2010; Lieschke and Currie, 2007).

Future directions

Gestationally survivable but clinically serious congenital cardiovascular defects can arise during mid to late embryonic development, but the human embryonic heart will obtain near-adult morphology before it can be imaged non-invasively via ultrasound(Hornberger et al., 1996; Lloyd-Jones et al., 2009). Safe methods to image the developing embryo may allow for earlier CHD detection and intervention. X-ray computed tomography (CT) and nuclear imaging methods such as positron emission tomography (PET) could provide the necessary resolution, but the contrast agents used for these imaging methods have an unknown fetal safety profile(Henning et al., 2011). Animal studies could provide the first steps to understanding the effects of contrast agents on cardiogenesis. Nanomaterials and nanoparticles are being investigated for use in a wide variety of applications including food and pharmaceutical applications(Chaudhry et al., 2008; Singh and Lillard Jr, 2009). The increased surface area, unique crystalline structure, small size, and enhanced reactivity of some nanomaterials may lead to cardiac developmental toxicity, and few studies have been performed to better understand nanoparticle interactions with embryonic development. Overall, there are still many unknowns surrounding the environmental, occupational, and pharmaceutical contributions to CHD, and this area should be the focus of major research efforts in the future.

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Table 1

Developmental age of early cardiac morphological events in different species. Adapted from Fishman and Chien (1997).

	Human	Mouse	Chick	Frog	Zebrafish
Migration of precardiac cells from epiblast	15-16 days	E7	HH4	Stage 10	5.5 hpf
First evident assembly of myocardial plate	18 days	E7	HH5	~Stage 13	~13 hpf
Generation of single heart tube initiated	22 days	E8	HH9	Stage 28	~19 hpf
Tubular heart starts contraction	23 days	E8.5	HH10	~Stage 33	22 hpf
Looping	23 days	E8.5	HH11	Stage 33-36	33 hpf
Cushions form	28 days	E9.5	HH17	~Stage 41	48 hpf

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Table 2

Exposures associated with definite or possible risk of offspring with congenital heart defects (CHD). Specific CHD are listed for exposures shown to produce a know defect. Table adapted from Jenkins et al (2007).

	Known defect(s)
Maternal illness	
Phenylketonuria	
Pregestational diabetes	Conotruncal defects
	Laterality and looping
	Dextro-looped transposition of the great arteries
	Atrioventricular septal defect
	Septal defects
	Hypoplastic left heart syndrome
	Outflow tract defects
	Patent ductus arteriosus
Febrile illness	Conotruncal defects
	Right-sided obstructive defects
	Tricuspid atresia
	Left-sided obstructive defects
	Aortic coarctation
	Ventricular septal defects
Influenza	Conotruncal defects
	Dextro-looped transposition of the great arteries
	Right-sided obstructive defects
	Left-sided obstructive defects
	Aortic coarctation
	Ventricular septal defects
	Dextro-looped transposition of the great arteries with intact ventricular septum
	Tricuspid atresia
Maternal rubella	Ventricular septal defects
	Patent ductus arteriosus
	Pulmonary valve abnormalities
	Peripheral pulmonic stenosis
Epilepsy	
Maternal therapeutic drug exposure	
Anticonvulsants	
Indomethacin tocolysis	Patent ductus arteriosus
Ibuprofen	Dextro-looped transposition of the great arteries
	Ventricular septal defects
	Bicuspid aortic valve
Sulfasalazine	

	Known defect(s)
Thalidomide	
Trimethoprim-sulfonamide	
Maternal nontherapeutic drug exposure	
Maternal vitamin A	Outflow tract defects
	Cranial neural crest defects (cardiac and noncardiac)
	Pulmonic stenosis
Marijuana	Ventricular septal defects
	Ebstein's anomaly
Maternal environmental exposure	
Organic solvents	Conotruncal defects
	Hypoplastic left heart syndrome
	Aortic coarctation
	Pulmonic stenosis
	Dextro-looped transposition of the great arteries with intact ventricular septum
	Tetralogy of Fallot
	Total anomalous pulmonary venous return
	Atrioventricular septal defect
	Ebstein's anomaly
	Ventricular septal defects

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL. 2007. Noninherited risk factors and congenital cardiovascular defects: Current knowledge. Circulation 115(23):2995-3014.

Table 3

Attributes of some key animals used to model human cardiac developmental toxicology. $, $$, $$$, $$$$ or *, **, ***, **** are the relative cost ($) or strength (*) of the model in each category.

	Animal model			
Attribute	**Mouse**	**Chick**	**Frog**	**Zebrafish**
Husbandry Costs	$$$$	$$	$	$
Laws and regulations regarding animal use	*	***	****	****
Anatomical similarity	***	***	**	*
Genetic similarity	***	**	*	*
Placental transfer of test compound?	yes	no	no	no
Maternal inactivation or bioactivation	yes	no	no	no
Embryonic Staging system?	yes	yes	yes	yes
Monitoring capability	*	***	****	****
Available molecular biology tools	****	**	**	***
Transgenic techniques available?	yes	no	yes	yes