

Chapter 2

In Vivo Models of Developmental Toxicology

Jason M. Hansen

Abstract

The founding principles of teratology/developmental toxicology state that a developmental toxicant cause dysmorphogenesis when conceptuses are exposed at a sufficient dosage during a sensitive period of development in a sensitive species. While in vitro approaches in developmental toxicology can provide a means to assess the potency of toxicants, ultimately, the need to use whole animal models to demonstrate embryotoxicity is necessary to fully extrapolate findings to the human condition. This chapter is dedicated to reviewing the advantages of specific animal models and how these animal models may be used to assess toxicity in the embryo, both descriptively and mechanistically.

Key words: Principles of teratology, In vivo, Animal models, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, Mouse

1. Introduction

Although studies over the last 100 years implicated environmental influences on development, teratology/developmental toxicology is a relatively new science. As a result, it was not until 1959 that James G. Wilson delineated six founding principles of teratology that really serve as a basis for all teratogenic studies (1). They are:

1. Sensitivity to teratogen-induced malformation depends on the genotype (species) of the conceptus.
2. Sensitivity to teratogen-induced malformation varies during different developmental stages at the time of exposure, where there are critical periods of sensitivity to agents and organ systems.
3. Teratogens act via a specific mechanism on developing cells and tissues to initiate a cascade of altered developmental events.
4. Teratogenic effects are dependent on the nature of the teratogen, including chemical properties of the chemical, route of exposure, maternal/fetal bioactivation, placental transport, etc.

5. Teratogens produce a consistent deviation from normal development. Deviation can include: (1) death, (2) malformation, (3) growth retardation, or (4) functional defect.
6. Teratogen-induced malformations occur in a dose-dependent manner, ranging from no observable defects to total lethality.

For proper characterization of suspected developmental toxicants, these principles must be testable and reproducible. In many cases, only whole animal models allow for the full analysis of teratogenicity/embryotoxicity.

Early work in developmental teratology focused on outcomes (i.e., the presence or absence of particular malformations), but lacked more mechanistic approaches. Many different in vitro approaches were developed to allow for more mechanistic experimentation. Many of these methods are described in this volume. However, while in vitro methods to assess developmental toxicology and embryo lethality have proven valuable, the fact remains that only in vivo testing can fully recapitulate the effects of a developmental toxicant, satisfying Wilson's six principles, and still remains a necessary part of developmental toxicology testing. Animal models allow for the assessment of developmental toxicity while preserving other toxicological aspects that are likely to be more relevant to human exposures.

Research in humans or in primates is severely limited due to ethical reasons and cost. Thus, a useful animal model would be relatively inexpensive and be easily manipulated experimentally. In the past 30 years, many different animal models have become readily available. Most of these were developed as a consequence of advances in genetics and developmental biology, but also lend themselves to be useful as models of developmental toxicology as well. Since many developmental pathways are conserved across a diversity of species, many of these animal models can be extrapolated to humans.

While the types of animals used in developmental toxicology studies are vast, for the purpose of this chapter, we focus on four distinct species: (1) the nematode, *Caenorhabditis elegans*, (2) the fruit fly, *Drosophila melanogaster*, (3) zebrafish, *Danio rerio*, and (4) the mouse, *Mus musculus*. A distinct advantage that these animal models share is their size, cost to maintain and rapid gestational periods compared to primate models, and although other species may also share these features, these four are distinct in that their genomes have been either fully or mostly mapped, allowing for mechanistic approaches to become more feasible through molecular biological methodologies. Table 1 summarizes some of these advantages and compares them to the same metrics found in humans (2).

Table 1

Comparative statistics from various animal models appropriate for developmental toxicology studies and from humans for comparison (Scientific frontiers)

Animal	Adult Size (cm)	Genome (Mb)	Organogenesis (days)	Generation time (weeks)
<i>Caenorhabditis elegans</i> (nematode)	0.1	97	0.2–0.4	0.4
<i>Drosophila melanogaster</i> (fruit fly)	0.4	180	0.5–1	2
<i>Danio rerio</i> (zebrafish)	3	1,700	1–4	12
<i>Mus musculus</i> (mouse)	6	3,000	6–15	10
<i>Homo sapiens</i>	170	3,500	14–60	1,400 (27 years)

1.1. *Caenorhabditis elegans*

C. elegans is a small round worm found in soil that has been of interest to biologists since the nineteenth century, mostly due to their simplicity. These small worms are easy to grow and have a short life cycle. Their development has been well characterized. In fact, during embryogenesis, a total of 671 cells are formed. The distinct advantage that *C. elegans* have is that their entire cell lineage has been established (3, 4). Of these cells, approximately 17% undergo apoptosis during progression toward adulthood. For that reason, much of the basic knowledge concerning apoptosis was originally worked out in *C. elegans* and now is quite well defined (5). Utility of this organism is increased as the entire genome has been sequenced (6) and have made the manipulation of gene expression more feasible. Overexpression approaches can be performed by injection of cloned genes into syncytium of the hermaphrodite gonad (7), where it recombines with DNA and is integrated into oocytes. Subsequently, offspring overexpress the gene of interest or introduce reporter. These approaches are a particular advantage as *C. elegans* is transparent. Thus, reporter constructs, such as GFP-constructs, are very useful. The discovery of essential embryogenic factors, such as Cathepsin L, and the germ layer differentiative patterning of *skn-1*-dependent genes were performed in embryos transfected with GFP constructs (8, 9). Conversely, the null phenotype can be achieved by the introduction of double-stranded mRNA, which interferes with endogenous gene function. Early experiments successfully inhibited expression of *unc22*, a nonessential myofilament gene, *fem-1*, a sex determining gene, and *hlh-1*, a *C. elegans* MyoD homolog (10).

Clearly, there are numerous advantages to the use of *C. elegans* as a model organism in developmental toxicology studies. Cost of maintenance is low, genetic manipulation is straightforward, and simplicity of the organism itself are all beneficial. Many important developmental pathways have been characterized in *C. elegans*, including the activation of programmed cell death through *ced-3*, *ced-4*, and *ced-9*, genes originally discovered in *C. elegans* (11). While many developmental breakthroughs have been made in *C. elegans*, its use in developmental toxicology has been somewhat limited. The most notable, potential problem with *C. elegans* as a toxicological model is extrapolation to human exposures. For the latter reason, *C. elegans* has been largely underutilized as a model of developmental toxicology. More information on *C. elegans* can be found in Chapter 3.

1.2. *Drosophila melanogaster*

D. melanogaster is commonly known as the fruit fly, whose genetics were studied extensively starting back in the 1920s in T. H. Morgan's laboratory at Columbia University. Use of *D. melanogaster* has led to many breakthroughs, including gene mapping, population genetics, and linkage. Because mutations can be easily viewed via microscopy *D. melanogaster* has been widely used as tools in understanding developmental/cellular biology processes. For example, much of the tyrosine kinase receptor pathways (i.e., *Ras*, *Raf*, guanine exchange factors [GEF], etc.) were well characterized in *D. melanogaster* systems, namely the *Sevenless*-related pathway. Because many developmental pathways that are present in *D. melanogaster*s may also be critical in higher vertebrates, they may also be a useful model of developmental toxicology.

*D. melanogaster*s are easy to grow and require very little equipment (i.e., milk bottles). Their generation period is short (only 2 weeks) and can be grown in large numbers inexpensively. Malformations can be easily detected under a microscopy. Perhaps one of the greatest strengths *D. melanogaster* (much like *C. elegans*) is its genome has also been mapped (12). However, because *D. melanogaster*s are invertebrates their utilization in developmental toxicological studies has been somewhat limited. However, *D. melanogaster*s were used in the assessment of methanol embryotoxicity. Exposure to methanol (4%) caused a variety of germ layer dysmorphogenesis and inhibited cell migration. Much like in vertebrates where alcohols target neurological systems during development, the CNS of germ band retracted *D. melanogaster* embryos, methanol caused an increase in apoptosis (13). Interestingly, embryos lacking programmed cell death genes (*grim*, *hid*, and *reaper*) showed less severe CNS defects, suggesting that methanol toxicity is mediated through apoptosis. These studies are an excellent example of the utility of *D. melanogaster* as model systems in developmental toxicology.

1.3. *Danio rerio* (Zebrafish)

Over the past 20 years, zebrafish are becoming a rapidly popular developmental model and are becoming more utilized for developmental toxicological studies. A chapter describing in detail zebrafish culture methodology is found in this volume (Chapter 4). Zebrafish share many of the previously discussed model system's advantages, they are easy to grow, organogenesis is well characterized and generation time is relatively short (compared to mammals); however, they are slightly more expensive to maintain, requiring setups that are not always readily available to some animal facilities. Their genome is mapped as well (14, 15). Perhaps one of the most attractive aspects of zebrafish as a model, unlike either the *C. elegans* or *D. melanogaster* models, is that they are vertebrates, and thus, developmental toxicology studies may be more applicable and relatable to human exposures. Zebrafish are easy to treat as they bioaccumulate chemicals that they are exposed to in their water. Embryos are transparent and embryonic morphology can be easily assessed throughout organogenesis. Zebrafish contain the same organ systems as do humans, unlike invertebrates. Of particular interest in developmental biology is the function of neural crest cells (NCC). The NCC are not found in invertebrates and since there are many developmental toxicants that can effect NCC development and function, zebrafish are good, inexpensive models to study developmental neurotoxicants. For example, a group in the Netherlands, used a large-scale assessment of ethanol developmental toxicity in zebrafish, where they used over 1,500 embryos, a feat that would not be reasonably feasible in mammal models (16). In zebrafish, ethanol induced pharyngeal arch hypoplasia, implicating the NCC as a target as they are involved in pharyngeal arch formation. Interestingly, in this study, at the time of ethanol treatment, NCC migration in zebrafish was complete, suggesting ethanol effects may occur postmigration, contrary to the hypothesis that ethanol affects NCC migration, as demonstrated in mouse and chick studies (17–19).

Morpholino technology can be used to effectively silence a gene of interest, thereby creating “knockouts” for testing purposes. This technology is explained in Chapter 5 of this volume in greater detail. This technology provides a powerful means to dissect pathways involved in differentiation and development. For example, vestigial and vestigial-like (VGLL) genes are expressed in facial prominences. In zebrafish, the ortholog *Vgll2a* is expressed in the pharyngeal endoderm and ectoderm surrounding NCC-derived mesenchyme of the pharyngeal arches. Using morpholino knock-down methodologies, *Vgll2a* expression was decreased and pharyngeal arch cell death increased, implicating *Vgll2a* in the development of the NCC-derived craniofacial structures (20). While “morpholino-like” manipulations are possible in higher vertebrates (i.e., knockout mice), cost and time can be prohibitive.

1.4. *Mus musculus* (Mouse)

Of all the animal models discussed here, the mouse is by far the most complex. Its genome size is approximately twice that of the zebrafish, measured to be 3,000 Mb (compared to 1,700 Mb in zebrafish), approaching the size and complexity found in humans. Since most human genes have mouse counterparts, mice may be best suited for studies that mimic toxicant exposures found in humans. Moreover, because the mouse genome is fully mapped (21), how toxic exposures affect gene expression and alter the function of specific transduction pathways make mechanistic approaches much more feasible to perform. Higher vertebrates, such as mammals, share certain characteristics that are lacking in some of the previously described models. These include: placentation, development in utero, and maternal/fetal metabolism and interactions. One of the distinct advantages that mice have over other mammalian models is transgenic manipulation, defined as the alteration of the genome by either addition of genetic material or by changing the existing gene by gene targeting. Many transgenic models are readily available through commercial sources and thus, are easily obtained by most laboratories. Transgenic technology has catapulted research toward more mechanistic endpoints that can be achieved in mammalian systems. Moreover, specific tissues from transgenic mouse embryos can be harvested and used in vitro for more molecular analyses. Some of these approaches are described in this volume (Brain and limb micromass in Chapter 9; Whole limb culture in Chapter 12; Whole embryo culture in Chapter 13).

The primary disadvantage in using mice for toxicological testing is cost and time. Compared to previously described animal models here, the number of mice needed to screen many chemicals at physiologically significant concentrations through many different routes of exposure is simply not possible. Up to 3,000 pups can be raised per year per square meter in stacked shelving in an approved animal facility, but by comparison, in the other animal models described here, proportional numbers can be achieved in less time and with less cost.

1.5. Other Animal Models

While we have highlighted these four animal models, it is critical to understand that there are other pertinent animal models available. Some of these include avian, frog, rabbit, rats, and others. In fact, many of these available animal models may be better for developmental toxicological studies and more relevant to human exposures. One of the founding principles listed above state delineates the importance of a susceptible genotype/species. Since animals are differentially sensitive to different toxicants, choosing the correct animal model for your study is critical. Nowhere is this better illustrated than with human thalidomide-induced teratogenicity. In this case, mice and rats are fairly insensitive to thalidomide-induced limb malformations, which led many to believe them safe for human consumption. However, thalidomide exposure in rabbit embryos caused

similarly limb manifestations (i.e., phocomelia) as was observed in humans (22). While thalidomide may be the most horrific example of human teratogenicity, nearly 45% of all known human teratogens were first identified in humans prior to being identified in animal models (23), indicating both a greater need of animal developmental toxicology testing and utilizing the correct model. Clearly, choosing the correct animal model for the specific toxicant in question is critical for both descriptive and mechanistic studies.

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