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Lower organisms as alternatives for toxicity testing in rodents

With a focus on *Ceanorhabditis elegans*
and the zebrafish (*Danio rerio*)

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Abstract

Lower organisms as alternatives for toxicity testing in rodents

With a focus on *Caenorhabditis elegans* and the zebrafish (*Danio rerio*)

The nematode *C. elegans* and the zebrafish embryo are promising alternative test models for assessment of toxic effects in rodents. Tests with these lower organisms may have a good predictive power for effects in humans and are thus complementary to tests with *in vitro* models (cell cultures). However, all described tests need further validation. This is the outcome of a literature survey, commissioned by the ministry of Health, Welfare and Sport of the Netherlands. The survey is part of the policy to reduce, refine and replace animal testing (3R policy).

In vitro models are considered as important alternatives for animal testing but lack the complexity of the human organism. Therefore, certain effects cannot be detected in *in vitro* models, such as indirect toxic effects or effects of compounds after chemical conversion to an active form in the whole organism. *C. elegans* and the zebrafish embryo can complement these shortcomings. These lower organisms can be considered as alternative test models because they do not experience inconvenience (such as pain) in the tests due to the low developmental state of their nervous system.

Tests with *C. elegans* and the zebrafish embryo are available for many types of toxicological research. Examples are the formation of tumours and toxic effects on reproduction, on the endocrine system, the heart, and the nervous system. A specific application of these organisms is in the safety assessment of new pharmaceutical products.

Key words:

hazard identification, 3R policy, alternatives for animal testing, lower organisms, *Caenorhabditis elegans*, zebrafish

Rapport in het kort

Lagere organismen als alternatief voor toxiciteitsonderzoek in knaagdieren

Met speciale aandacht voor *Caenorhabditis elegans* en de zebravis (*Danio rerio*)

De rondworm *C. elegans* en het embryo van de zebravis zijn veelbelovende alternatieve testmodellen voor onderzoek naar schadelijke effecten van chemische stoffen in knaagdieren. Proeven met deze lagere organismen hebben naar verwachting een goede voorspellende waarde voor effecten in de mens en zijn daarmee een zinvolle aanvulling op testen met *in-vitro*modellen (celcultures). Dit blijkt uit een inventariserend literatuuronderzoek, uitgevoerd in opdracht van het ministerie van VWS. Het onderzoek is onderdeel van het beleid om dierproeven te verminderen, te vervangen of te verfijnen (het zogeheten 3V-beleid). Nader onderzoek naar de validatie van de testen moet nog plaatsvinden.

*In-vitro*modellen gelden als een belangrijk alternatief voor dierproeven maar missen de complexiteit van het menselijk organisme. Hierdoor kunnen *in-vitro*modellen bepaalde effecten niet signaleren, zoals indirecte schadelijke effecten van stoffen of effecten ervan nadat ze in het organisme zijn omgezet en daardoor een andere structuur hebben gekregen. De lagere organismen *C. elegans* en het zebravisembryo kunnen deze tekortkomingen overbruggen. Bovendien ervaren ze geen ongerief, zoals pijn, vanwege de lage graad waarin hun zenuwstelsel is ontwikkeld.

Testen met *C. elegans* en het zebravisembryo kunnen voor veel soorten toxicologisch onderzoek worden gebruikt. Voorbeelden zijn de vorming van tumoren en schadelijke effecten van stoffen op de voortplanting, het hormoonstelsel, het hart en het zenuwstelsel. Bovendien zijn experimenten gaande waarbij deze organismen worden gebruikt in het veiligheidsonderzoek van nieuwe geneesmiddelen.

Trefwoorden:

toxiciteitsonderzoek, 3V-beleid, alternatieven voor dierproeven, lagere organismen, *Caenorhabditis elegans*, zebravis

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Summary

This report summarizes the major literature on the perspectives of the use of lower organisms as alternatives for toxicity testing of chemicals in rodents, with a focus on the nematode *Caenorhabditis elegans* and the zebrafish (*Danio rerio*) embryo. Current *in vitro* models (cell cultures) which were developed as alternatives for animal tests in toxicology are limited in their predictive value for adverse health effects in humans, because cell cultures cannot model the complexity of a whole organism. Therefore, indirect effects, long term effects, effects requiring interaction between multiple systems, and effects that require metabolic modification of a potentially toxic agent, can be missed in an *in vitro* model. Lower organisms do not have these drawbacks and could thus provide a useful addition to the test batteries that are developed as alternatives for animal tests. The use of lower organisms implies a refinement of animal tests because due to the more primitive organisation of their nervous system, lower organisms do not perceive pain or otherwise discomfort.

Using these two organisms, a large number of tests are available, which together cover a wide range of toxicological fields, such as carcinogenesis and mutagenesis, effects in reproduction, in the endocrine and nervous systems, and in the heart. Furthermore, trials are going on with use of these organisms in safety assessment of pharmaceuticals.

In both *C. elegans* and the zebrafish embryo, the application of novel molecular techniques is well developed. These techniques enhance the potential for detailed description of toxicological effects and optimal comparison with effects in *in vitro* systems and conventional animal models. Furthermore, such techniques enable the design of sensitive models for specific end points.

In conclusion, the nematode *C. elegans* and the zebrafish embryo are promising models for implementation as alternatives for animal testing in toxicity assessment. However, the step towards formal validation for acceptance in human risk assessment has not been taken with any of the developed tests with these organisms, and this should be encouraged urgently.

1 Introduction

While there is a demand to test a growing list of chemicals to estimate their effects on human and environmental health, the use of traditional mammalian models for toxicological testing is associated with scientific, economic, and ethical concerns. The need to reduce, refine or replace mammalian organisms in toxicological testing is therefore imposed by regulatory agencies world-wide, for instance as expressed by the European Commission (European Commission, 1986, 2008), the US National Toxicology Program (NTP) and the US Environmental Protection Agency (EPA), (Becker et al., 2006). The use of nonmammalian whole organism models has been defined as a possible strategy to relieve some of the pressure to test ever more compounds while reducing the number of mammals used. Development of such alternative testing models is supported by recent advances in genetic technologies and the detection of strong conservation between human and nonmammalian genomes which allow for the dissection of the molecular pathways involved in toxicological responses (and diseases) using genetically tractable organisms. It has now become abundantly clear that some non-mammals are convenient materials, because toxicological assays based on these organisms are more rapid and less expensive than traditional mammalian-based tests due in part to shorter life spans and the ability to assay in multi-well plate formats. Welfare concerns in non-mammalian organisms also differ from those in mammals. On the other hand, non mammalian organisms are also endowed with physiological and pharmacological properties common to humans. Thus, several such organisms have become very popular alternative organisms and are being used extensively as models. This report presents two examples: *Caenorhabditis elegans* (*C. elegans*) and embryos of *Danio rerio* (*D. rerio*, zebrafish). For each of these organisms, several toxicological tests are being developed, implementing current knowledge on genetics, biochemistry, physiology and pharmacology in these organisms.

2 *C. elegans*

This chapter contains citations from Jacobs, 2006; Hoss and Weltje, 2007; Tyl et al., 2007; Leung et al., 2008; Peterson et al., 2008.

C. elegans is a saprophytic nematode organism, inhabiting soil and leaf-litter environments in many parts of the world (Hope, 1999) and often carried by terrestrial gastropods and other small organisms in the soil habitat (Caswell-Chen et al., 2005; Kiontke and Sudhaus, 2006). *C. elegans* has a number of features that make it a relevant and powerful model for biological research. First of all, *C. elegans* is easy and inexpensive to maintain in laboratory conditions with a diet of *Escherichia coli*. The short, hermaphroditic life cycle (~3 days) and large number (300+) of offspring of *C. elegans* allows large-scale production of animals within a short period of time (Hope, 1999). Since *C. elegans* has a small body size (1 mm), *in vivo* assays can be conducted in a 96-well microplate. The transparent body with a simple and well characterized anatomy allows clear observation of all cells in mature and developing animals. Furthermore, the genome of *C. elegans* has been sequenced and contains approximately 20,000 genes, and about 35 % of these have human homologs. Significant advantages of using *C. elegans* for genetic analysis is the opportunity to incorporate forward and reverse genetic screens to relatively quickly identify proteins and molecular pathways that are involved in particular cellular processes. Forward genetic screens using chemical mutagens have the advantage that no a priori knowledge is needed to determine a gene's function in order to determine whether it plays a role in the particular phenotype. Mutations can be identified within as little as a week (Wicks et al., 2001), and since the worms can easily be mated with each other, strains containing several mutations can be generated within days (Wicks et al., 2001). *C. elegans* is also amenable to reverse genetics that allows for the identification of molecular pathways in which a particular gene acts upon. RNA interference, or RNAi, where the introduction of dsRNA induces sequence specific degradation of homologous mRNAs and subsequent protein expression, is one example of a reverse genetic approach; genome-wide RNAi screens are now routinely used in forward genetic approaches. Thus, the intensively studied genome, complete cell lineage map, knockout (KO) mutant libraries, and established genetic methodologies including mutagenesis, transgenesis, and RNA interference (RNAi) provide a variety of options to manipulate and study *C. elegans* at the molecular level (Leung et al., 2008).



Figure 1 - Laboratory culture of *C. elegans*, showing eggs, various larval stages, and adult worms (size of adult worms is approx. 1 mm). By courtesy of Dr. Hendrik Korswagen, Hubrecht Institute, Utrecht NL.

As a whole-organism model, *C. elegans* provides several characteristics that complement *in vitro* or cellular models. The use of whole-organism assays, first of all, allows the study of a functional multicellular unit, such as a serotonergic synapse, instead of a single cell (Kaletta and Hengartner, 2006). *C. elegans* also enables the detection of end points at the level of the organism (*e.g.*, feeding, reproduction, life span, and locomotion) and the interaction of a chemical with multiple targets in an organism. Thus, *C. elegans* complements both *in vitro* and *in vivo* mammalian models in toxicology. Of note, these characteristics facilitate high-throughput experiments that can examine both fundamental toxicity, which are critical since so many chemicals have yet to be thoroughly tested, and the gene-gene and gene-environment interactions whose importance is just beginning to be appreciated in toxicology.

C. elegans has been recognized as an attractive biological and genetic model organism for some time (Brenner, 1974). Recently, the above mentioned advantages have led to a rise in the use of *C. elegans* as a toxicity test organism. Short life cycles, easy and inexpensive maintenance, and detailed biological knowledge allow for the development of rapid, low-cost tests that readily lend themselves to mechanistic studies of toxicant action. There is also a high degree of conservation in the molecular toxicological responses between *C. elegans* and mammals. For example, many signal transduction pathways involved in general stress responses are well conserved (Committee on Developmental Toxicology, 2000). Several studies have also demonstrated the predictive potential of *C. elegans* lethality and changes in locomotion for mammalian toxicity (Tatara et al., 1998; Cole et al., 2004). With advances in technology, the assessment of phenotypes of thousands of nematodes can now be quantified in a high-throughput fashion, rather than by direct observation of only a few organisms (Peterson et al., 2008).

Currently, *C. elegans* is explored as a model organisms in several fields of toxicology. The paragraphs below focus on assays with relevance for human health. While some tests can be applied to ecotoxicology as well, that chapter is not explicitly considered here.

2.1 Carcinogenesis and mutagenicity

The integrity of the genome is essential to the health of the individual and to the reproductive success of a organisms. Transmission of genetic information is in a selective balance between two opposing forces, the maintenance of genetic stability versus elimination of mutational change and loss of evolutionary potential. *C. elegans* provides many advantages for the study of DNA surveillance and repair in a multicellular organism. Several genes have been identified by mutagenesis and RNA interference that affect DNA damage checkpoint and repair functions. Many of these DNA damage response genes also play essential roles in DNA replication, cell cycle control, development, meiosis and mitosis. To date, a DNA damage-induced response has been identified in the *C. elegans* in germ line, which is characterized by two spatially separate checkpoints; mitotic germ nuclei proliferation arrest and apoptosis of damaged meiotic nuclei. Both of these responses are regulated by checkpoint genes including *mrt-2*, *hus-1*, *rad-5* and *cep-1*, the *C. elegans* ortholog of the human tumour suppressor p53. The germ line DNA damage checkpoints in *C. elegans* provide an excellent model to study the genes required to maintain genomic stability and to test compounds which might have tumor suppressing properties. In addition to single gene studies, integration of data from high-throughput screens has identified genes not previous implicated in the DNA damage response and elucidated novel connections between the different repair pathways. Most of the genes involved are conserved between worms and humans, and in humans, are associated with either oncogenesis or tumor-suppression (O'Neil and Rose, 2006).

Mutation is a central biological process whose rates and spectra are influenced by a variety of complex and interacting forces. Although DNA repair pathways are generally known to play key roles in maintaining genetic stability, much remains to be understood about the relative roles of different pathways in preventing the accumulation of mutations and the extent of heterogeneity in pathway-specific repair efficiencies across different genomic regions. Study of mutation processes in base excision repair-deficient (*nth-1*) and nucleotide excision repair-deficient (*xpa-1*) included generation of *C. elegans* mutation-accumulation (MA) lines across 24 regions of the genome and comparison to data from mismatch repair-deficient (*msh-2* and *msh-6*) and wild-type (N2) MA lines. Drastic variation in both average and locus-specific mutation rates was detected among the four sets of repair-deficient MA lines. The relative roles of three DNA repair pathways in preventing *C. elegans* mutation accumulation and the presence of pathway-specific DNA repair territories in the *C. elegans* genome have been elucidated (Denver et al., 2006), and several repair pathway reporter strains are now available (Van Haaften et al., 2004; Kruisselbrink et al., 2008).

Studies of the physical and functional interactions of the components of the repair pathways in *C. elegans* are providing information about human repair disorders and cancer predisposition, and enable assessment of toxic effects of xenobiotic compounds on these pathways.

2.2 Reproduction toxicology

C. elegans matures from fertilized egg to adult through four distinct larval stages, termed L1–L4, in approximately 3.5 days and has an average life span of 10 days.

As the nematodes grow and develop, they increase in length and optical density. Using fluorescent worms, these parameters can be measured in automated toxicological assays, which have been developed to assess chemical sensitivity at specific developmental stages: L1s for growth, and L4s for reproduction. In the *C. elegans* growth assay, length and optical density from L1s are measured at the beginning of the assay and then after a 72 h exposure to the test toxicant. The *C. elegans* reproduction assay was developed to assess the effects of chemicals at the L4 stage, just before nematodes are reproductive. After 48 h exposures, the number of offspring is counted. In all assays, dose–response curves are calculated to estimate toxicological endpoints such as EC50s and benchmark doses. The growth and reproduction assays were validated by screening almost 60 chemicals, which include metals, pesticides, mutagens, and non-toxic agents, and further high throughput screens with 1408 chemicals are projected (Boyd and Freedman in Peterson et al., 2008).

In the future, studies with *C. elegans* may have more use as part of a screen for developmental effects by drug sponsors. The NTP has undertaken a large research programme on the use of *C. elegans* in the evaluation of possible developmental toxicants, with a variety of end points, including the use of microarrays to assess drug impact on gene expression (Jacobs, 2006).

2.3 Neurotoxicology

Of the over 1000 cells of *C. elegans*, approximately one-third is identified as neurons. The nervous system of *C. elegans* is highly conserved with mammals, and contains almost all of the known signaling and neurotransmitter systems found in the vertebrates (Bargmann, 1998; Nass and Blakely, 2003). The transparency of the nematode and the ease of making transgenic animals containing reporter constructs greatly facilitates examination of neuron protein expression and localization (Mello and Fire, 1995; Thomas and Lockery, 1999). The high sequence similarity between human and *C. elegans* genomes allows for the dissection of the molecular pathways involved in neurological diseases using this model organism. The nematode has therefore been recognized as a powerful genetic model for exploring the molecular mechanisms of neuron function and human disease (Riddle et al., 1997; Nass et al., 2001), as particularly applied to identify and characterize genes and proteins involved in Parkinson's disease (PD) and its related toxicological syndrome manganism. Similarly, this worm has the potential for identifying novel proteins and compounds that may be involved in, or protect against, neurodegenerative disorders, and it can also be utilized to screen and identify neurotoxic environmental agents.

In PD research, etiological and pathological data suggest that there is both a genetic and environmental component that causes and contributes to the dopamine (DA) neuron cell death (Jenner, 1998).

Environmental exposure to manganese, as well as several other metals, has been associated with the development of PD. Mn-induced Parkinsonism, also called manganism, has been associated with Mn mining and welding (Chia et al., 1993; Calne et al., 1994).

A common mechanism to model Parkinson's disease in vertebrates is through exposure of the animals to a DA neuron neurotoxin, such as 6-hydroxydopamine (6-OHDA), which is transported into the cell by the high affinity dopamine transporter, DAT. Brief exposure of the worms to 6-OHDA (0.5–1 h) induces a time and concentration-dependent loss of DA neuron integrity, as visualized in a *C. elegans*

PD model by expressing GFP in DA neurons (Nass et al., 2002). Also, this effect can be blocked by co-incubation with DAT agonist (e.g. amphetamine) or antagonist (e.g. cocaine), consistent with these vertebrate models of PD that DAT is required (Nass et al., 2002). These results mimic several significant aspects of the vertebrate PD model, and should allow to identify potential environmental components such as Mn²⁺ exposure and novel endogenous molecules that could contribute to the DA neuron cell death. Transgenic worms with deficits in the pathways associated with PD, such as overexpression of α -synuclein, which is involved in DA biosynthesis and transportation, may even show amplified sensitivity for these environmental toxicants.

Thus, *C. elegans* provides remarkable opportunities to identify and characterize genes and proteins involved in Parkinson's disease and manganism *in vivo*. The high similarities on the molecular level between the worm and humans suggest that the paradigms discovered using this system are highly relevant to screen and identify environmental agents such as heavy metals that could affect the DA system (Nass in Peterson et al., 2008)

Other assays to screen for neurotoxicity using *C. elegans* include (automated) movement and feeding assays. In the feeding assay, nematodes are exposed to toxicants for 24 h (Boyd et al., 2007). After exposures, nematodes are allowed to feed on small fluorescent microspheres and the level of fluorescence in individual nematodes is measured. Feeding rates of exposed and non-exposed control *C. elegans* can then be calculated. Movement assays are based on exposure induced effects using semi-automated motion tracking.

In the future, these and similar assays with *C. elegans* may have more use as part of a screen for neurotoxic effects by drug sponsors. Studies have been conducted using *C. elegans* in the evaluation of behavioural neurotoxicity (Anderson et al., 2004), as *C. elegans* responds to various neurotransmitters. The NTP has undertaken a large research programme on the use of *C. elegans*, in the evaluation of possible neurological toxicants, with a variety of end points, including the use of microarrays to assess drug impact on gene expression (Jacobs, 2006).

2.4 Endocrine disruption

Although an endocrine system is not known in nematodes, there is evidence that many processes are regulated via hormonal pathways. It is considered that vertebrate hormones, such as steroids, may have endocrine functions in nematodes as well, and chemicals which have endocrine disrupting activity in vertebrates also appear to affect nematodes on all organizational levels, from molecules to communities. *C. elegans* is considered as a promising organism for the development of biomonitoring tools for endocrine disruption, although there is a need for more mechanistic evidence on hormonal processes within these organisms (Hoss and Weltje, 2007), as well as on the implication of differences between toxicant's target receptors between mammals and non-mammals.

Some examples of genetically modified *C. elegans* models with relevance to endocrine disruption testing are knockout of cytochrome P450 35A subfamily showing that CYP35A is required for fat storage and resistance to PCB52 toxicity (Menzel et al., 2007) and the bis-1 mutant created from EMS mutagenesis yielded collagen mutants which are hypersensitive to bisphenol-A (Watanabe et al., 2005).

2.5 Drug development and drug safety

Although in individual cases *C. elegans* may be used to evaluate environmental effects or bridge between different lots of materials, further data and evaluation are necessary in order to understand the strengths and limitations, sensitivity and specificity of using the nematode for more than screening assays in the discovery phase of pharmaceutical development. Studies in worms may find greater use when toxic effects can be linked to genetic events or particular signalling pathways. What is learned from NTP initiatives with these organisms may be informative for pharmaceuticals in the future. Important issues regarding deliberate pharmaceutical use in humans include the potential human adverse effects at particular exposures, the safe first dose and the highest reasonable dose in humans, acceptable toxicity for humans at the particular stage of drug development, reversibility of identified adverse effects, important toxicological endpoints for a new compound, and safety at pregnancy and at long-term use. The applicability of the *C. elegans* model in the evaluation of the nonclinical safety of pharmaceuticals will increase with the number of such issues that can be addressed with the model (Jacobs, 2006).

2.6 Conclusion

In conclusion, several toxicological assays using *C. elegans* have been developed or are currently under development. Transgenic strains to monitor toxicological processes include metal response, biotransformation, apoptosis, and DNA damage response. Further development of green fluorescent protein-based and other transgenic *C. elegans* models will improve the sensitivity and specificity of toxicity screens and enable high throughput testing. *C. elegans* is a promising candidate organisms for implementation as an alternative testing model in a wide variety of toxicological disciplines. However, no *C. elegans* based assays have been validated or implemented for testing for human toxicity, nor are such efforts going on. The use of the *C. elegans* model in defined expert areas, i.e. carcinogenesis, mutagenesis, endocrine disruption, liver and reproduction toxicology should be further explored.

3 Zebrafish embryos

This chapter contains citations from Jacobs, 2006; Peterson et al., 2008; Piersma et al., 2009.

The zebrafish (*Danio rerio*) is a small cyprinid fish of the Indian subcontinent with an adult length of approximately 4 to 5 cm (Laale, 1977). The organisms is easily maintainable in the laboratory, shows a high fecundity under appropriate conditions, and completes its life-cycle within three months. The zebrafish has been a major model in biomedical research for several decades, resulting in an immense body of information on organisms development and molecular embryology (Nusslein-Volhard, 1994; Lele and Krone, 1996; Dooley and Zon, 2000). The morphology of embryonic development of zebrafish, which has been described in great detail (Kimmel et al., 1995), can be followed real-time due to the transparency of the eggs and embryos (Figure 2).

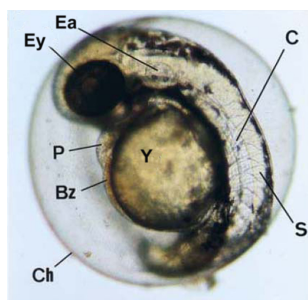


Figure 2 - Zebrafish embryo 48 hours post fertilization, illustrating transparency of egg and embryo, enabling assessment of major structures. Ey, eye anlage; Ea, ear; S, somites; C, chorda; Y, yolk sac; P, pericardium. Egg diameter is approximately 0.6 mm.

Further advantages of the zebrafish embryo model are its short embryonic period (48 hours organogenesis) and the minimal space and test volume requirements: toxicity testing with zebrafish embryos can be conducted using a single well of a 96- or 384-microtiter plate per embryo. Zebrafish small molecule screening takes advantage of the small size, chemical permeability, and optical transparency of the zebrafish. Embryonic and larval stages of the zebrafish can be exposed to small molecules by adding the compounds to the water in the wells, and the effects can be observed in the transparent embryos using microscopy. Finally, large similarities exist in early development and the signaling repertoire between organisms in general (Arthur, 2002), and between zebrafish and mammals specifically (Schilling and Webb, 2007).

According to the Directive 86/609/EEC and its revision (see above), embryos are not defined as experimental animals, and are, as such, not subject to animal welfare regulations. This view was confirmed by the European Food Standards Agency (EFSA) Scientific Panel on Animal Health and Welfare (EFSA Scientific Panel on Animal Health and Welfare, 2005). In addition, EFSA stated that the embryo should be regarded as an experimental animal from the stage onward where the embryo is capable of feeding independently rather than being dependent on egg yolk. This is in agreement with formally considering ZFET as an experimental animal-free alternative toxicity test. In the United States, there is no uniform position as to whether fish embryos are regulated as test organisms. The US Animal Welfare Act (AWA) applies to warm-blooded animals (USA Government, 2007) but further

regulations may depend on the testing facility's funding source and accreditation status. In this way, regulations may apply for zebrafish offspring only after hatching in studies funded by the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (US NIH, 2009), or for zebrafish at any phase of life in laboratories under accreditation of The Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, which is a voluntary program for animal research laboratories in the US (AAALAC, 2009).

3.1 Environmental toxicity

Fish embryo toxicity tests were originally developed in the context of environmental toxicity testing, with a focus on lethality endpoints. Braunbeck and Lammer (Braunbeck and Lammer, 2006) recently reviewed the existing database on fish embryo toxicity tests, as a basis for discussions on the potential of Zebrafish embryotoxicity test (ZFET) as an alternative for acute toxicity testing in adult fish. Lethality endpoints at 48 hr post fertilization is scored as coagulation of the embryo, no detachment of the tail, absence of heart-beat and of somite formation. Other easily observed developmental and teratogenic endpoints are spontaneous movement, heart-beat (frequency), blood circulation, pigmentation, formation of edemata, malformations of head/face/arches/jaw, sacculi/otoliths, tail, heart, liver, GI tract; modified chorda structure/scoliosis/rachitis/tail/fins, yolk deformation, and general growth retardation (illustrated in Figure 3). These all can be scored discretely in a standardized way (Nagel, 2002; Busquet et al., 2008).

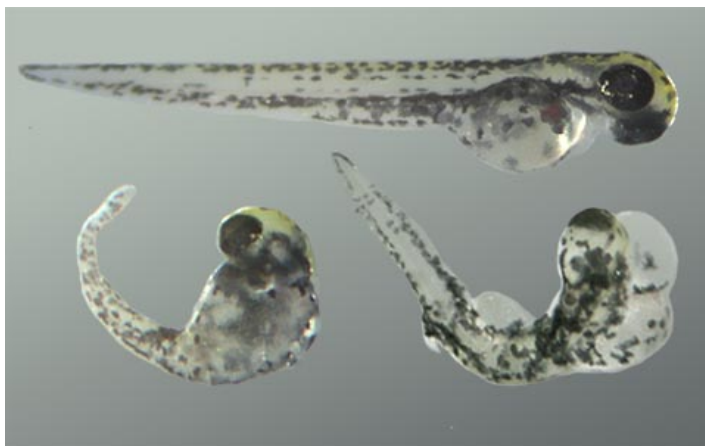


Figure 3 - Severe mixed teratogenic effects of propylthiouracil in zebrafish embryos (lower specimen, compare with upper age-matched control) (described in Van der Ven et al., 2006)

Sensitivity of morphological assessment can be increased using specific techniques, such as Nomarski imaging. Additional simple endpoints are in the behavioral domain, such as spontaneous movement and touch response (normal-twitch-spasm-nonresponse) and can be recorded and analyzed in an automated way.

Formal validation of the ZFET as an alternative for acute toxicity testing in fish is going on as an activity in the OECD Validation Management Group for Non-Animal testing.

3.2 Reproduction toxicology

Whereas zebrafish embryos have been used for decades to elucidate the mechanisms of developmental toxicity, with TCDD and ethanol as mostly studied reference compounds, this model was more recently also identified for potential screening of developmental toxicants (Mirkes et al., 2003; HESI, 2007; HESI-ECETOC, 2008).

The zebrafish is sensitive for disruption of developmental processes, with conserved teratogenic mechanisms compared with humans (Rubinstein, 2006; Kari et al., 2007). The most appealing advantages of this model are that effects can be evaluated in a whole organism and at an early developmental stage by virtue of transparency of eggs and embryos; the specificity of effects due to high similarity of detected effects and of modes of action of toxicants between zebrafish and mammals; the availability of sophisticated genetic tools; and cost-effectiveness of the model. These advantages make zebrafish a useful model organisms for routine embryotoxicity testing.

The teratogenic potency may coincide with or separate from that of lethality effects (Figure 4). Differentiation between such effects may be important for the interpretation of the assay. Because the hatched embryo appears to be more sensitive to toxic effects of some chemicals than the prehatched embryo, separation of the eleutheroembryonic stage (between hatching and independent feeding) from the prehatch stage may be useful; however, this difference can be explained by the relative impermeability of the outer eggshell layer, the chorion. Techniques are available to avoid this barrier, particularly mechanical and/or enzymatic dechoriation or microinjection of the egg (Braunbeck et al., 2005).

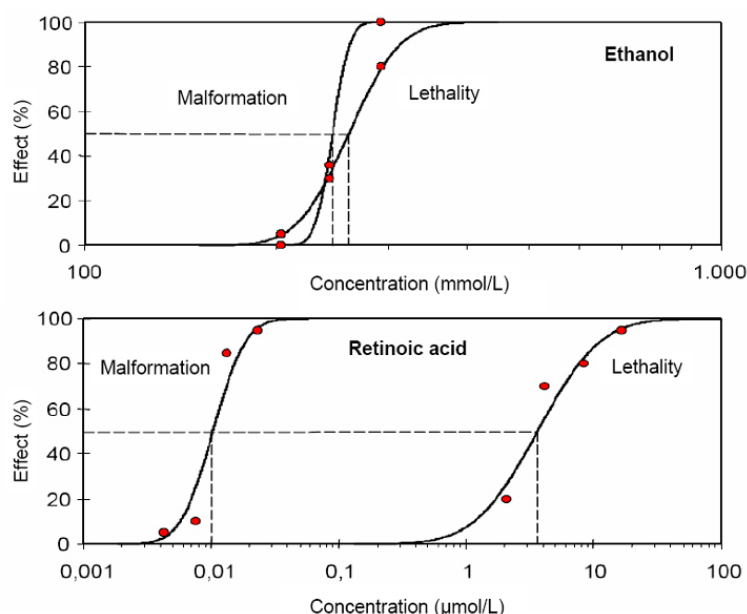


Figure 4 - Teratogenicity (morphological malformation) versus acute lethality in zebrafish embryos, with similar (ethanol, upper panel) or different (retinoic acid, lower panel) sensitivity for these endpoints (from: Bachmann, 2002; with permission).

A major advantage in the context of developmental toxicity testing is that zebrafish orthologues of human CYP enzymes have been identified (Rubinstein, 2006). These metabolic enzymes become progressively functional during zebrafish embryogenesis (Rubinstein, 2006; Amali et al., 2006), and this early metabolic competence is compatible with the early development of a fully functional digestive system; however, combined exposure of proteratogens with a metabolic activation system may increase the sensitivity of the test when an early exposure window is applied (Busquet et al., 2008). Variant protocols developed in multiple labs showed similar outcomes, indicating the robustness of the model. For global acceptance, standardization is imperative. For this purpose, allelic diversity between strains, ambient conditions, egg dechoriation, delivery of compounds with low solubility (solvent or microinjection), stress responses, and exposure and assessment window need to be considered.

Although no formal validation of ZFET for toxic effects in mammals, such as developed by ECVAM (Worth and Balls, 2001), has yet been performed, several reports have assessed the predictivity of zebrafish embryo tests for mammals. A study in zebrafish embryos with 12 pharmaceutical compounds presented a 91% success rate in positively identifying teratogenic and non-teratogenic compounds in rodents (Seng and Augustine, 2007). In a study with 6 known human teratogens, the test with zebrafish appeared equally predictive (83 %) to rodents (Ali, 2007). These results may even improve when molecular tools are applied (Yang et al., 2007). Differences in developmental gene expression repertoire between organisms may affect predictivity.

Applying the ZFET for more detailed developmental toxicity testing may require the inclusion of additional morphological endpoints and application of molecular tools. Since the zebrafish genome is well-defined, there is good availability of a wide variety of molecular tools (Zon and Peterson, 2005). In this respect, good promoters are available for the production of stable transgenic fish which can be generated with high efficiency. Transgenic models can be enhanced by using reporters through fluorochromes that reflect timely gene expression and its disruption, such as GFP coupled AhR regulated GFP expression to detect TCDD activation (Huuskonen, 2005). Furthermore, there is availability of genome-wide screens, and the genomic response was suggested to provide a more sensitive readout than morphological effects, with specificity of toxicogenomic responses for tested toxicants (Yang et al., 2007; Van Rompay et al., 2008). These techniques allow the identification of developmentally controlled genes and signaling pathways, and thus improved databases are being generated for mapping of target mutations to specific phenotypes (on/off target effects, mode of action). Zebrafish provide opportunities for stringent control design by way of antisense morpholino technique, while (siRNA) inhibition of gene function can alter the frequency of developmental disorders (Voelker et al., 2008). Implementing such higher tier molecular tools may further enhance the applicability of the ZFET for developmental toxicity testing, make it suitable for cost effective high-throughput testing, and deliver specific markers for specific effects.

3.3 Cardiac toxicology

Zebrafish are amenable to high-throughput screening in small molecule discovery and cardiac toxicology. Zebrafish small molecule screening takes advantage of the small size, chemical permeability, and optical transparency of the zebrafish embryo. Transgenic lines expressing fluorescent proteins in specific neuronal subpopulations have also been developed, which could facilitate screening. Cardiotoxicity is perhaps the most thoroughly tested zebrafish toxicity to date. Zebrafish screens have also been used to discover novel compounds that suppress the effects of genetic vascular defects (Peterson et al., 2008).

Much work remains to be done to determine the extent to which zebrafish toxicities are predictive for humans, but initial data from cardiotoxicity are encouraging. In an assay for drug-induced bradycardia, 22 of 23 compounds known to cause human QT prolongation were detected among 100 tested compounds, suggesting a high degree of correlation between zebrafish and human cardiotoxicity (Milan et al., 2003).

3.4 Neurotoxicology

The first small molecule screens performed employed wild-type zebrafish and visual screening to identify obvious morphological defects (Peterson et al., 2000; Sternson et al., 2001; Spring et al., 2002; Moon et al., 2002; Khersonsky et al., 2003; Shafizadeh et al., 2004; Peterson et al., 2008). These screens identified defects in numerous organ systems including the central nervous system (CNS). Phenotypes identified in this way were generally severe, and for the CNS ranged from loss or expansion of brain ventricles to truncation of the telencephalon to severe neuronal necrosis. While these studies demonstrate the ability of this approach to identify small molecules that cause severe developmental neurotoxicities, it is doubtful that such screens could reliably identify subtle neurotoxicities that are not manifest in obvious morphological changes. More sophisticated assays will likely be necessary. Vital dyes like acridine orange have been reported to stain apoptotic cells in zebrafish and may help detect subtle neurotoxicities (Parng et al., 2004). Transgenic lines expressing fluorescent proteins in specific neuronal subpopulations have also been developed, which could facilitate screening. Numerous functional and behavioral assays, including assays of vision, hearing, touch responsiveness, memory, anxiety, and startle habituation have been developed and could also be useful for identifying neurotoxicants that do not cause obvious developmental phenotypes (Brockerhoff et al., 1995; Fetcho et al., 1998; Bang et al., 2002; Peitsaro et al., 2003). It is possible that a panel of several high-throughput morphological and functional assays could be used to screen broadly for neurotoxicants.

Increasing the number and sophistication of high-throughput neuronal assays for zebrafish will be of little value if zebrafish and human neurotoxicities do not correlate. Validation studies as indicated above (see cardiac toxicology), focused on neurotoxicity would be very useful but have not been reported. However, some individual compounds have been reported to have predictable neurotoxicities in zebrafish, including ethanol, 6-hydroxydopamine, acrylamide, MPTP, and pentylenetetrazole (McKinley et al., 2005; Baraban et al., 2005; Parng et al., 2007).

3.5 Drug development and drug safety

Zebrafish have been used as a screen for drug- or chemical-related toxicity and safety for drugs that will be given to humans. However, it is not clear how much tests for developmental toxicity can be relied on for other than screening studies. Zebrafish have not been used for pivotal toxicology studies submitted to the US FDA for pharmaceuticals. So far, for pharmaceuticals regulated by the US FDA, zebrafish have been used for nonclinical assessment of efficacy of one drug product (as measured by an 85 % decrease in neuronal apoptosis) and for an environmental assessment of effects (short-term toxicity) of another drug product. In the ecotoxicity study, the drug being reviewed was shown to have a toxicity profile equivalent to an already approved drug. A third product was evaluated for effects on zebrafish embryonic development. One interpretation for lack of expected effects in that study was the possible inability of that particular test compound to enter the differentiating cells (Jacobs, 2006). The National Toxicology Programme (NTP) plans to use zebrafish to help set testing priorities (NTP, 2004) and has funded research in the area.

3.6 Conclusion

In conclusion, in the search for alternative models for toxicity testing, the zebrafish offers much: low cost, high throughput, an almost limitless range of morphological and functional assays, and an apparently high degree of correlation with mammalian systems. What is missing is history. Unlike many mammalian models that have been used for decades, the zebrafish cannot benefit from a large reservoir of historical data establishing the system's validity and limitations. If the zebrafish is to become useful, it will require a commitment to accumulating and sharing that reservoir, a process that hopefully could be accelerated by the ability to acquire data rapidly in zebrafish. Although such an effort would be formidable, the competing pressures for additional testing and reduced use of mammals may indicate that an investment in zebrafish is a sound one (Peterson et al., 2008). The use of the zebrafish embryo model in defined expert areas, *i.e.* carcinogenesis, mutagenesis, endocrine disruption, liver and reproduction toxicology, should therefore be further explored.

4 Perspectives

A vast number of tests has been developed using *C. elegans* and zebrafish embryos to detect toxic effects relevant to humans. Tests based on these organisms have the advantage of considering the complexity of a whole organism, in contrast to tests based on cultured cells. Therefore, tests with these lower organisms can complement *in vitro* tests. However, until now, none of these tests has been formally validated for use in human hazard assessment. An example of pilot studies aiming at such validation is the initiative by HESI/ILSI to implement a teratogenicity test with zebrafish embryos in a test battery for reproduction toxicology.

Support to initiate formal implementation of the many potentially useful tests based on *C. elegans* and zebrafish embryos in test batteries and to their acceptance for human hazard assessment could contribute to a major reduction of the use of mammals in toxicity testing.

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References

- AAALAC, 2009. <http://www.aaalac.org/accreditation/rules.cfm>.
- Ali, N., 2007. Teratology in zebrafish embryos: a tool for risk assessment. In. Swedish University of Agricultural Sciences, Uppsala.
- Amali, A.A., Rekha, R.D., Lin, C.J., Wang, W.L., Gong, H.Y., Her, G.M., Wu, J.L., 2006. Thioacetamide induced liver damage in zebrafish embryo as a disease model for steatohepatitis. *J.Biomed.Sci.* 13, 225-232.
- Anderson, G.L., Cole, R.D., Williams, P.L., 2004. Assessing behavioral toxicity with *Caenorhabditis elegans*. *Environ.Toxicol.Chem.* 23, 1235-1240.
- Arthur, W., 2002. The emerging conceptual framework of evolutionary developmental biology. *Nature* 415, 757-764.
- Bachmann, J., 2002. Entwicklung und Erprobung eines Teratogenitäts-Screening Testes mit Embryonen des Zebrafisblings *Danio rerio*. In. Technischen Universität Dresden.
- Bang, P.I., Yelick, P.C., Malicki, J.J., Sewell, W.F., 2002. High-throughput behavioral screening method for detecting auditory response defects in zebrafish. *J.Neurosci.Methods* 118, 177-187.
- Baraban, S.C., Taylor, M.R., Castro, P.A., Baier, H., 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* 131, 759-768.
- Bargmann, C.I., 1998. Neurobiology of the *Caenorhabditis elegans* genome. *Science* 282, 2028-2033.
- Becker, R.A., Borgert, C.J., Webb, S., Ansell, J., Amundson, S., Portier, C.J., Goldberg, A., Bruner, L.H., Rowan, A., Curren, R.D., Stott, W.T., 2006. Report of an IS RTP workshop: progress and barriers to incorporating alternative toxicological methods in the U.S. *Regul.Toxicol.Pharmacol.* 46, 18-22.
- Boyd, W.A., McBride, S.J., Freedman, J.H., 2007. Effects of genetic mutations and chemical exposures on *Caenorhabditis elegans* feeding: evaluation of a novel, high-throughput screening assay. *PLoS.ONE.* 2, e1259.
- Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-organisms -- an update. *ALTEX.* 22, 87-102.
- Braunbeck, T., Lammer, E., 2006. Fish Embryo Toxicity Assays. University of Heidelberg / German Federal Environment Agency, Heidelberg / Dessau.
- Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.
- Brockhoff, S.E., Hurley, J.B., Janssen-Bienhold, U., Neuhauss, S.C., Driever, W., Dowling, J.E., 1995. A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc.Natl.Acad.Sci.U.S.A* 92, 10545-10549.
- Busquet, F., Nagel, R., von Landenberg, F., Mueller, S.O., Huebler, N., Broschard, T.H., 2008. Development of a new screening assay to identify proteratogenic substances using zebrafish *danio rerio* embryo combined with an exogenous mammalian metabolic activation system (mDarT). *Toxicol.Sci.* 104, 177-188.
- Calne, D.B., Chu, N.S., Huang, C.C., Lu, C.S., Olanow, W., 1994. Manganism and idiopathic parkinsonism: similarities and differences. *Neurology* 44, 1583-1586.
- Caswell-Chen, E.P., Chen, J., Lewis, E.E., Douhan, G.W., Nadler, S.A., Carey, J.R., 2005. Revising the standard wisdom of *C. elegans* natural history: ecology of longevity. *Sci.Aging Knowledge.Environ.* 2005, e30.
- Chia, S.E., Foo, S.C., Gan, S.L., Jeyaratnam, J., Tian, C.S., 1993. Neurobehavioral functions among workers exposed to manganese ore. *Scand.J.Work Environ.Health* 19, 264-270.
- Cole, R.D., Anderson, G.L., Williams, P.L., 2004. The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. *Toxicol.Appl.Pharmacol.* 194, 248-256.

- Committee on Developmental Toxicology, B.o.E.S.a.T.C.o.L.S.N.R.C., 2000. Signaling Pathways (Appendix C). In: Scientific frontiers in developmental toxicology and risk assessment. National Academy Press, Washington, DC, pp. 296-308.
- Denver, D.R., Feinberg, S., Steding, C., Durbin, M., Lynch, M., 2006. The relative roles of three DNA repair pathways in preventing *Caenorhabditis elegans* mutation accumulation. *Genetics* 174, 57-65.
- Dooley, K., Zon, L.I., 2000. Zebrafish: a model system for the study of human disease. *Curr.Opin.Genet.Dev.* 10, 252-256.
- EFSA Scientific Panel on Animal Health and Welfare, 2005. Opinion on the 'Aspects of the biology and welfare of animals used for experimental and other scientific purposes'. *The EFSA Journal* 292, 1-46.
- European Commission, 1986, 2008. Directive 86/609/EEC and its revision; http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm.
- Fetcho, J.R., Cox, K.J., O'Malley, D.M., 1998. Monitoring activity in neuronal populations with single-cell resolution in a behaving vertebrate. *Histochem.J.* 30, 153-167.
- HESI, 2007. <http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3334>.
- HESI-ECETOC, 2008. <http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3438>.
- Hope, I.A., 1999. Background on *Caenorhabditis elegans*. In: Hope, I.A. (Ed.), *C. elegans: a practical approach*. Oxford University Press, New York, pp. 1-15.
- Hoss, S., Weltje, L., 2007. Endocrine disruption in nematodes: effects and mechanisms. *Ecotoxicology* 16, 15-28.
- Huuskonen, H., 2005. New models and molecular markers in evaluation of developmental toxicity. *Toxicol.Appl.Pharmacol.* 207, 495-500.
- Jacobs, A., 2006. Use of nontraditional animals for evaluation of pharmaceutical products. *Expert.Opin.Drug Metab Toxicol.* 2, 345-349.
- Jenner, P., 1998. Oxidative mechanisms in nigral cell death in Parkinson's disease. *Mov Disord.* 13 Suppl 1, 24-34.
- Kaletta, T., Hengartner, M.O., 2006. Finding function in novel targets: *C. elegans* as a model organism. *Nat.Rev.Drug Discov.* 5, 387-398.
- Kari, G., Rodeck, U., Dicker, A.P., 2007. Zebrafish: an emerging model system for human disease and drug discovery. *Clin.Pharmacol.Ther.* 82, 70-80.
- Khersonsky, S.M., Jung, D.W., Kang, T.W., Walsh, D.P., Moon, H.S., Jo, H., Jacobson, E.M., Shetty, V., Neubert, T.A., Chang, Y.T., 2003. Facilitated forward chemical genetics using a tagged triazine library and zebrafish embryo screening. *J.Am.Chem.Soc.* 125, 11804-11805.
- Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. *Dev.Dyn.* 203, 253-310.
- Kiontke, K., Sudhaus, W., 2006. Ecology of *Caenorhabditis* organisms. *Wormbook* January 09, 1-14.
- Kruisselbrink, E., Guryev, V., Brouwer, K., Pontier, D.B., Cuppen, E., Tijsterman, M., 2008. Mutagenic capacity of endogenous G4 DNA underlies genome instability in FANCD1-defective *C. elegans*. *Curr.Biol.* 18, 900-905.
- Laale, H.W., 1977. The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. A literature review. *J.Fish Biol.* 10, 121-173.
- Lele, Z., Krone, P.H., 1996. The zebrafish as a model system in developmental, toxicological and transgenic research. *Biotechnol.Adv.* 14, 57-72.
- Leung, M.C., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., Meyer, J.N., 2008. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. *Toxicol.Sci.* 106, 5-28.
- McKinley, E.T., Baranowski, T.C., Blavo, D.O., Cato, C., Doan, T.N., Rubinstein, A.L., 2005. Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. *Brain Res.Mol.Brain Res.* 141, 128-137.

- Mello, C., Fire, A., 1995. DNA transformation. *Methods Cell Biol.* 48, 451-482.
- Menzel, R., Yeo, H.L., Rienau, S., Li, S., Steinberg, C.E., Sturzenbaum, S.R., 2007. Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode *Caenorhabditis elegans*. *J.Mol.Biol.* 370, 1-13.
- Milan, D.J., Peterson, T.A., Ruskin, J.N., Peterson, R.T., MacRae, C.A., 2003. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* 107, 1355-1358.
- Mirkes, P., McClure, M.E., Heindel, J.J., Sander, M., 2003. Developmental toxicology in the 21st century: multidisciplinary approaches using model organisms and genomics. *Birth Defects Res.A Clin.Mol.Teratol.* 67, 21-34.
- Moon, H.S., Jacobson, E.M., Khersonsky, S.M., Luzung, M.R., Walsh, D.P., Xiong, W., Lee, J.W., Parikh, P.B., Lam, J.C., Kang, T.W., Rosania, G.R., Schier, A.F., Chang, Y.T., 2002. A novel microtubule destabilizing entity from orthogonal synthesis of triazine library and zebrafish embryo screening. *J.Am.Chem.Soc.* 124, 11608-11609.
- Nagel, R., 2002. DarT: The embryo test with the Zebrafish *Danio rerio*--a general model in ecotoxicology and toxicology. *ALTEX.* 19 Suppl 1, 38-48.
- Nass, R., Miller, D.M., Blakely, R.D., 2001. *C. elegans*: a novel pharmacogenetic model to study Parkinson's disease. *Parkinsonism.Relat Disord.* 7, 185-191.
- Nass, R., Hall, D.H., Miller, D.M., III, Blakely, R.D., 2002. Neurotoxin-induced degeneration of dopamine neurons in *Caenorhabditis elegans*. *Proc.Natl.Acad.Sci.U.S.A* 99, 3264-3269.
- Nass, R., Blakely, R.D., 2003. The *Caenorhabditis elegans* dopaminergic system: opportunities for insights into dopamine transport and neurodegeneration. *Annu.Rev.Pharmacol.Toxicol.* 43, 521-544.
- NTP, 2004. <http://ntp-server.niehs.nih.gov/files/06291.pdf>.
- Nusslein-Volhard, C., 1994. Of flies and fishes. *Science* 266, 572-574.
- O'Neil, N., Rose, A., 2006. DNA repair. *Wormbook*, 1-12.
- Parng, C., Anderson, N., Ton, C., McGrath, P., 2004. Zebrafish apoptosis assays for drug discovery. *Methods Cell Biol.* 76, 75-85.
- Parng, C., Roy, N.M., Ton, C., Lin, Y., McGrath, P., 2007. Neurotoxicity assessment using zebrafish. *J.Pharmacol.Toxicol.Methods* 55, 103-112.
- Peitsaro, N., Kaslin, J., Anichtchik, O.V., Panula, P., 2003. Modulation of the histaminergic system and behaviour by alpha-fluoromethylhistidine in zebrafish. *J.Neurochem.* 86, 432-441.
- Peterson, R.T., Link, B.A., Dowling, J.E., Schreiber, S.L., 2000. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc.Natl.Acad.Sci.U.S.A* 97, 12965-12969.
- Peterson, R.T., Nass, R., Boyd, W.A., Freedman, J.H., Dong, K., Narahashi, T., 2008. Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology* 29, 546-555.
- Piersma, A.H., Van Dartel, D.A.M., van der Ven, L.T., 2009. Alternative Methods in Developmental Toxicology. In: Knudsen, T.B., Daston, G.P. (Eds.), *Developmental Toxicology*. Elsevier.
- Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R., 1997. *C. elegans* II. Cold Spring Harbor Laboratory Press, New York.
- Rubinstein, A.L., 2006. Zebrafish assays for drug toxicity screening. *Expert.Opin.Drug Metab Toxicol.* 2, 231-240.
- Schilling, T.F., Webb, J., 2007. Considering the zebrafish in a comparative context. *J.Exp.Zoolog.B Mol.Dev.Evol.* 308, 515-522.
- Seng, W.L., Augustine, K.A., 2007. Zebrafish: a predictive model for assessing developmental toxicity. In: HESI Workshop on alternative assays for developmental toxicity, Cary, N.C.
- Shafizadeh, E., Peterson, R.T., Lin, S., 2004. Induction of reversible hemolytic anemia in living zebrafish using a novel small molecule. *Comp Biochem.Physiol C.Toxicol.Pharmacol.* 138, 245-249.

- Spring, D.R., Krishnan, S., Blackwell, H.E., Schreiber, S.L., 2002. Diversity-oriented synthesis of biaryl-containing medium rings using a one bead/one stock solution platform. *J.Am.Chem.Soc.* 124, 1354-1363.
- Sternson, S.M., Louca, J.B., Wong, J.C., Schreiber, S.L., 2001. Split--pool synthesis of 1,3-dioxanes leading to arrayed stock solutions of single compounds sufficient for multiple phenotypic and protein-binding assays. *J.Am.Chem.Soc.* 123, 1740-1747.
- Tatara, C.P., Newman, M.C., McCloskey, J.T., Williams, P.L., 1998. Use of ion characteristics to predict relative toxicity of mono-, di- and trivalent metal ions: *Caenorhabditis elegans* LC50s. *Aquat.Toxicol.* 42, 255-269.
- Thomas, J.H., Lockery, S.H., 1999. Neurobiology. In: Hope, I.A. (Ed.), *C. elegans: a practical approach*. Oxford University Press, New York, pp. 143-179.
- Tyl, R.W., Sloan, T.S., Hamby, B.H., Ehman, K.D., Sumner, S., 2007. EPA Draft Hershberger background review document. U.S. Environmental Protection Agency, Endocrine Disruptor Screening Program.
- US NIH, 2009. http://grants.nih.gov/grants/olaw/faqs.htm#App_4.
- USA Government, 2007. http://www.aphis.usda.gov/animal_welfare/publications_and_reports.shtml.
- Van der Ven, L.T., van den Brandhof, E.J., Vos, J.H., Power, D.M., Wester, P.W., 2006. Effects of the antithyroid agent propylthiouracil in a partial life cycle assay with zebrafish. *Environ.Sci.Technol.* 40, 74-81.
- Van Haaften, G., Plasterk, R.H., Tijsterman, M., 2004. Genomic instability and cancer: scanning the *Caenorhabditis elegans* genome for tumor suppressors. *Oncogene* 23, 8366-8375.
- Van Rompay, A., Selderslaghs, I., Hooyberghs, J., Hollanders, K., Boonen, F., Geukens, G., Witters, H., 2008. Gene expression signatures in zebrafish embryos: an alternative model for developmental toxicity. In: CARDAM-VITO / I-Sup2008, Brugge.
- Voelker, D., Stetefeld, N., Schirmer, K., Scholz, S., 2008. The role of cyp1a and heme oxygenase 1 gene expression for the toxicity of 3,4-dichloroaniline in zebrafish (*Danio rerio*) embryos. *Aquat.Toxicol.* 86, 112-120.
- Watanabe, M., Mitani, N., Ishii, N., Miki, K., 2005. A mutation in a cuticle collagen causes hypersensitivity to the endocrine disrupting chemical, bisphenol A, in *Caenorhabditis elegans*. *Mutat.Res.* 570, 71-80.
- Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H., Plasterk, R.H., 2001. Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat.Genet.* 28, 160-164.
- Worth, A.P., Balls, M., 2001. The importance of the prediction model in the validation of alternative tests. *Altern.Lab Anim* 29, 135-144.
- Yang, L., Kemadjou, J.R., Zinsmeister, C., Bauer, M., Legradi, J., Muller, F., Pankratz, M., Jakel, J., Strahle, U., 2007. Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. *Genome Biol.* 8, R227.
- Zon, L.I., Peterson, R.T., 2005. *In vivo* drug discovery in the zebrafish. *Nat.Rev.Drug Discov.* 4, 35-44.

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