Teratology in Zebrafish Embryos: A Tool for Risk Assessment

Nadeem Ali

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Nadeem Ali

Division of Pathology, Pharmacology and Toxicology
Department of Department of Biomedical Sciences and Veterinary Public Health
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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of aquatic toxicology.

Nadeem Ali,

Division of Pathology, Pharmacology and Toxicology
Department of Department of Department of Biomedical Sciences and Veterinary Public Health
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences (SLU)
P.O. Box 7028, SE- 750 07 Uppsala, Sweden
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To my family

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Abbreviations

RA	Retinoic acid
6-AN	Aminonicotinamide
OA	Ochratoxin A
FAS	Foetal alcohol syndrome
CNS	Central nervous system
DNA	Deoxyribonucleic acid,
T-RNA	Transfer Ribonucleic acid
ANOVA	Analysis of variance
NOEC	No Observed Effect Concentration
LOEC	Lowest Observed Effect Concentration
HPF	Hours post fertilization
DMSO	Dimethyl Sulfoxide
M	Molar

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"In the name of God, most Gracious, most Compassionate".

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"My peers accept me and respect me, and that's enough."

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Abstract

Teratology, study of abnormal prenatal development, as a descriptive science has starts with written language. The modern experimental teratology era started in the early quarter of 20th centaury. Since the thalidomide catastrophe in early 1960s regulatory agencies launched requirements for new drugs to be thoroughly tested on animals prior to human use. One of the major concerns in the teratological studies is the mechanism of teratogenesis; it is very difficult to know the exact mechanism of teratogenesis. However there are many proposed mechanism of teratogenesis by Wilson 1973. Teratogens induce one or multiple unique pathogenic responses in the developing embryos. Susceptibility f teratogenesis varies with age and therefore can be divided into three developmental periods: early embryonic development, organogenesis and early differentiation, and late embryonic development.

Animal based studies provide the initial guideline if a chemical or drug may present a teratogenic risk. A variety of laboratory animals from different classes of animals are being used for the teratological studies. Rat, rabbit, mice, hamster, and non human primates are the most prevalent laboratory animal species of the mammal class. Xenopus laevis of the amphibian class has been used and suggested as a model for mammalian teratogenicity. From the bird class chicken, duck and quail have been used most often in laboratory studies. Zebrafish, Japanese medaka and fathead minnow are the most commonly used laboratory fish species, promoted by OECD for future testing of chemical toxicity. Teratogens can be classified as recreational and social teratogens, pharmaceutical teratogens, industrial and environmental teratogens, agricultural teratogens, and metabolic and infectious diseases. In the present study model substances were selected from the different classes of teratogens. The selected substances were; retinoic acid, lithium, ethanol, 6-aminonicotinamide, ochratoxin A and arsenic,

.

Key words: teratology; teratogenesis; retinoic acid; lithium; ethanol; 6-aminonicotinamide; ochratoxin A ; arsenic; zebrafish

Background

Historical background of teratology

Teratology is the study of abnormal prenatal development and congenital malformations, which may be caused by exposure to chemicals or physical factors. Teratology as a descriptive science starts with written language, a marble sculpture from southern Turkey dating back to 6500 B.C., depicts conjoined twins (Warknay,1983), and Egyptian wall paintings of human malformed conditions such as cleft palate and achondroplasia have been dated as early as 5000 years ago. The Babylonians Greek and Romans believed that abnormal infants were reflections of stellar events and such were considered to be portents of future. The Latin word monstrum from monstrare (to show) or monere (to warn) is derived from this perceived ability of malformed infants to foretell the future. In turn root of teratogenesis is from Greek word teraton meaning "wonder" and by derivation "monster" (Francis, 1994).

Modern experimental teratology as a science was born in 1920s and 1930s, when the birth of malformed piglets from sows fed an experimental diet high in fat or deficient in vitamin A elicited shocking teratogenic effects (Hale, 1933; Schardein 1993). Subsequent evidence about teratology came to light over the next two decades; correlation of particular birth defects of children with maternal Rubella infection in 1941 (Gregg, 1941) and with environmental mercury contamination in 1956 (Igata, 1993); malformed rat born following the inclusion of a growth inhibiting amino acid mimic in their mother's diet (Murphy et al., 1956) and malformed children born following failed Aminoptern induced abortions (Thiersh, 1956).

Thalidomide represents the 1st case of a substance producing minimal toxicity in the adult but considerable toxicity in the foetus (MC Bride, 1961; Lenz, 1966). Since the Thalidomide catastrophe regulatory agencies launched requirements for new drugs to be thoroughly tested on animals prior to approval for marketing (Bailey et. al., 2005; Rowan, 1984).

The confounding nature of results from experiences based on animal studies has required principles to be elaborated and revised (Wilson, 1997; Finnell, 1999). Many variables have been found to interfere with interspecies and animal human comparisons (Nielsen et al., 2001; Palmer, 1986), these must be considered when designing developmental and reproductive toxicology studies. They can be summarized as;

I. Susceptibility to teratogenesis depends on the genotype of the conceptus and how it interacts with the environment (Schardein, 1993).
II. Susceptibility to a teratogenic agent varies with the developmental stage at which the exposure occurs (Wilson, 1972).
III. Teratogenic agents act in specific ways on developing cells and tissues to initiate abnormal embryogenesis (Wilson, 1973).

IV. Access of an adverse environmental agent to developing tissues depends on the nature of the agent (Polifka and Friedman, 1999).
V. The manifestations of deviant development increase in degree as dosage increase from the co-effect to the lethal level (Brent, 1995).
VI. Manifestation of deviant development includes death, malformation and growth retardation. (Wilson ,1972).

Animal based studies of developmental toxicology provide the initial information's on whether a drug or chemical constitute a teratogenic risk substance. Typically, a range of doses administered via the most appropriate route is given to pregnant animals during the period of embryonic organogenesis and the outcomes are compared to untreated control animals. The most prevalent species used are mice, rats, hamster, primates and rabbits but no one species is an ideal experimental animal because they are not fulfilling the "Ideal" criteria such as producing large litters after a short gestation, inexpensive maintenance and an inability and unwillingness to' bite, scratch , kick, howl or squeal' (Wilson, 1975). It is also acknowledged that the laboratory handling of animals can induce physiological stress responses and cause alterations in behaviour and both these factors can affect teratogenicity results. Participation in the teratology research itself is so inherently stressful that this can never be excluded (Balcombe et al., 2004).

During the past 30-40 years a lot of money has been spent on teratological testing, but still scientist are looking for alternative laboratory species which can produce large litter after a short gestation, easy and inexpensive handling, inability and unwillingness to' bite, kick, scratch , howl or squeal' (Wilson, 1975).

Mechanism of Teratogenesis

Mechanism of teratogenesis falls into two broad categories based on the etiology of the congenital malformation:

a. Errors in the genetic programming based on deviations in the genotype of the embryo or the low probability for errors of a normal genotype.
b. Environmental agents or factors that interact with the embryo during the period of development, e.g., drugs, chemicals, radiation, infections or mechanical factors.

Etiology of malformations includes both genetic and environmental factors, but there is another large category labelled as unknown, e.g., polygenic, multifactorial, spontaneous errors of development and synergistic interactions of teratogens.
To induce malformations, teratogens must cross the placenta or reach the developing embryo through some other route, which makes phenomenon of teratogenesis applicable to all organisms including those in which embryonic development occurs outside the mother.

A number of suggested mechanisms involved in teratogenesis has been proposed by Wilson (1973);

1. Mutation: Changes in the nucleotide sequence in DNA. However, mutation is unlikely to play a role in the production of malformations following in utero exposure. Mutagens usually produce cytotoxic effects which are related to the cell destruction not to the genetic changes that persist and effect embryonic development for many cell cycles in the developing embryo.
2. Chromosomal aberrations: alterations in the amount of DNA. However, chromosomal aberrations play little or no role when induced in somatic cells of the developing embryo but may cause cell death and retardation of differentiation.
3. Altered nucleic acid synthesis and function: a disturbance in translation, transcription or DNA synthesis.
4. Mitotic interference: a disturbance in the cell cycle.
5. Lack of precursors, substrates and co-enzymes for biosynthesis: a general or specific nutritional deficiency.
6. Altered energy source: interference with the citric acid cycle or terminal electron transport system.
7. Enzyme inhibition: limited or specific enzyme inhibition.
8. Osmolar imbalance: alterations in the fluid pressures, viscosities and osmotic pressures.
9. Altered membrane characteristics: a disruption in membrane transport and permeability.
10. Other mechanisms: an extensive list of possible mechanisms for which there is a little scientific support.

In the developing embryo many of the above mentioned cellular insults can cause unique pathogenic responses (Tyl, 2000) such as:

1. Reduced cell proliferation.
2. Cell death.
3. Altered cell-cell interactions
4. Reduced biosynthesis.
5. Mechanical disruption of developing structures.
6. Inhibitions of morphogenetic movements.

Susceptibility of teratogenesis with age

Susceptibility to teratogenesis varies with the developmental stage (F1) (Tyl, 2000). It is generally accepted that every teratogen acts at specific times of development, and that these vary, within limits, between agents. The limits are determined by the developmental sequences of the embryo. The stages of gestation can be divided, at first approximation, into 3 developmental periods: Early embryonic development, organogenesis, and late embryonic development.

1. **Early embryonic development;** the embryo consists of relatively few cells, which are still totipotent. This means that if they are moved to a different site on/in the embryo, they will become what their new position dictates. A teratogen which causes significant disruption of the embryo in early embryonic development is usually lethal. Less drastic degrees of cell death can be compensated, especially in the mammalian embryo, which obtains nutrients through maternal transfer.

2. **Organogenesis and early differentiation;** this period is characterized by rapid growth and differentiation of most organ systems. Extremely rapid cell proliferation make the embryo particularly susceptible to teratogens, since any decrease in nutrient levels, as well as cytotoxicity, can lead to reduced cell number. Moreover, the increasing number of cells permits survival of the embryo even after significant damage. The period of organogenesis is the time when most structural teratogens induce their effect.

3. **Late embryonic development;** this period is marked by growth, maturation and functional differentiation of the organs formed in the previous stage. Interference with development at this period leads to more subtle structural malformations and to functional deficits, of which the most dramatic is probably mental retardation. Functional deficits can be induced in most organs even in the last trimester. The brain continues to develop throughout prenatal life and for some time after birth, which means that mental retardation can be induced from postnatal exposures, e.g., from the maternal milk. Growth retardation may also be a consequence of disturbances during this phase of development.

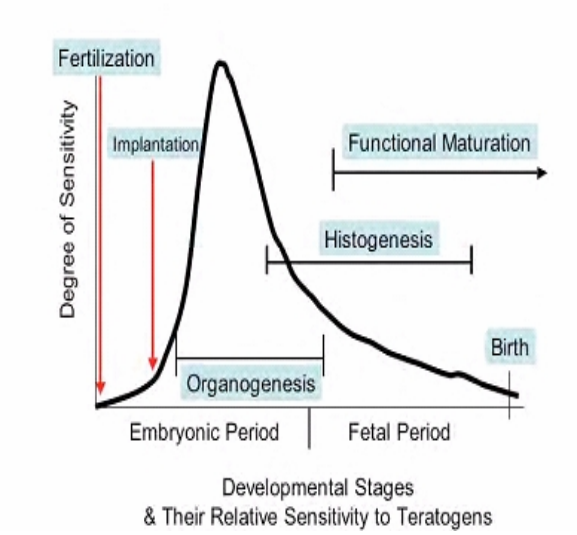

Figure 1. Relationship between developmental stage and sensitivity to teratogenesis (from Tyl, 2000).

Laboratory animals for teratogen testing

Animal-based studies of developmental toxicology provide the initial information on whether a drug or chemical may present a teratogenic risk. Typically, a range of doses administered via the most appropriate route is given to pregnant animals during the period of embryonic organogenesis, and the outcomes compared to control untreated animals. The most prevalent species used are mice, rats and rabbits. The usual sample size is 20 individuals per dose, and the dose range is selected so that the highest dose causes signs of toxicity, the lowest causes no discernable effect in the foetus, and at least one intermediate dose.

Considering, physiological and biochemical differences between animal species used in teratology research and humans, means that it is impossible for a single species to have all desirable properties. Desired characteristics for the ideal teratology animal have been proposed in a 'wish list', but no species comes close to fulfilling the criteria. For example, no species absorbs, metabolises and eliminates test substances like a human nor possesses the same placental transfer properties; no one species has the same pre-term developmental and metabolic patterns as humans; and, even if it were possible to meet all of these standards, the animal would be unlikely to meet other 'ideal' criteria such as producing large litters after a short gestation, inexpensive maintenance, and an inability and unwillingness to 'bite, scratch, kick, howl or squeal' (Wilson, 1975).

Mammals

Rodents have become the most commonly used group for evaluating potential human teratogens. Proponents of animal use, while admitting that it can only give an approximation of effects in humans, praise the rat model since for many years all human teratogens identified exhibited teratogenesis in rats (Tuchmann-Duplessis et al., 1972). There are, however, important exceptions, such as with the prostaglandin E1 analogue misoprostol: treatment of humans with this drug for peptic ulcer disease or to initiate labour has a strong association with foetal malformations known as Moebius syndrome (Pastuszak et al., 1998), but is not teratogenic in the rat even up to 10 times the human dose (Klasco and Heitland, 2003). There are many examples of positive results in rodents that have little or no effect in humans ('false positives'), especially at normal exposures and therapeutic dose levels. Notable examples include glucocorticoids and benzodiazepines, which induce oral clefts in rats, mice and rabbits, but not in humans (Baxter and Fraser, 1950; Buresh and Urban, 1970; Czeizel 1987; Fainstat, 1954; Fraser and Sajoo, 1995; Miller and Becker, 1975; Pinsky and DiGeorge, 1965; Rosenberg et al., 1983; Shepard 1994; Shiono and Mills, 1984; Walker 1971; Wilson et al., 1970). Also aspirin, which causes cardiac malformations in several species of animals, such as the rat and the rhesus monkey, is harmless in man (Beall and Klein, 1977; Klein et al., 1981; Slone et al., 1976; Werler et al., 1989; Wilson et al., 1977).
Because of the inherent problems and inadequacies of teratology testing and research with the five groups of animals most commonly used (mouse, rat, rabbit,

hamster, and monkey), scientists have tried to incorporate other species into their experiments in an attempt to find something approaching that elusive 'ideal animal'. Dogs (more specifically beagles) were tested with an array of known teratogenic compounds, but deemed unsuitable due to poor sensitivity; in any case, it is known that many drugs are metabolised differently in the dog, and there are particular problems with extrapolation from dogs to humans with reference to steroids (Schardein, 1993). By using cats there was some promising concordance with several compounds, though in common with dogs they are known to metabolise a significant number of drugs differently, some uniquely, and there were discordant results compared to other species with compounds such as hydantoins, thalidomide, and especially the anti-leukaemia drug and abortifacient aminopterin, which is highly teratogenic in humans but not at all in cats (Schardein, 1993). Pigs were found to be as insensitive as dogs, ferrets did not live up to early expectations, and non-human primates, despite their close phylogenetic relationship to humans, have been particularly disappointing as a predictive model (Schardein, 1993). Over 100 teratogenic agents classified as 'possible' or 'probable' have been tested in nonhuman primates, and the vast majority showed a high level of discordance; of the known human teratogens tested, only about half were found also to be teratogenic in one or more primate species (Bailey et. al., 2005).

Amphibians

Amphibians have proven to be useful tools in both classical and modern embryology. The African clawed frog, Xenopus laevis, has also been suggested as a model for mammalian teratogenicity. Amphibian development is also used to identify overall aquatic toxicity and the chemicals used for such studies tend to be highly relevant to ecological problems. Xenobiotics known to affect development in amphibians include methyl mercury (5 to 30 ppb), high levels of selenium, the fungicides dichlone and chloranil and benzo (a) pyrene.

Birds

Birds have proven important in all aspects of developmental biology and toxicology. Chick embryos are excellent experimental subjects for embryologic investigations, since they are easily obtained, can be maintained outside the egg for at least 24 hours, and are large enough for easy manipulation. The effect of DDT in thinning eggshells (Ratcliffe, 1967) and of PCBs in causing malformations in birds, have been documented repeatedly. Recently, selenium emerged to induce developmental malformations in birds (Ratti et al., 2006). Chicken, duck and quail have been used most often in laboratory studies, but correlations between pollutants in eggs and reproductive problems in wild birds have been carried out in numerous species ranging from gulls to cormorants and ospreys. It is quite clear that there is as much variability among avian as among mammalian species in response to reproductive and development toxicants. Chlorinated hydrocarbon insecticides, PCBs, and selenium, many or most organophosphate insecticides (Ops) are potent teratogens in birds. The avian

teratogenicity of organophosphates is in striking contrast to their inactivity in mammalian development. Lead on the other hand, appears to be less toxic to avian than to mammalian development.

Fish

Development in fish has been studied because of the economic importance of many species and because fish are used as indicators in connection with environmental assessment. Zebrafish, Japanese medaka, goldfish, rainbow trout and fathead minnow are the most common laboratory species, which also are key species promoted by OECD for future testing of chemical toxicity.

The zebrafish, Danio rerio, has been used extensively as a model species for developmental biology. This species is small in size (3-5cm in length), easily obtainable, inexpensive, readily maintainable, under appropriate conditions will provide a large number of non-adherent and transparent eggs (Laale, 1997). One female lays approximately 50 to 200 eggs per day. The morphological and molecular basis of tissues and organ development in zebrafish are in general either identical or similar to other vertebrates including men (Chen and Fishman, 1996; Granato and Nusslein-volhard, 1996). There is extensive similarity between the zebrafish and human genomes so many human developmental and disease genes have counterparts in the zebrafish. The zebrafish genome is 1700 million base pairs in length, about half the size of the human genome. Most human genes have homologues to zebrafish and the functional domain of the protein such as ATP binding domain of kinases are almost 100% identical between homologous genes, although the similarity over the entire protein is about 60% (Langheinrich, 2003). As protein function largely resides in functional domains where drugs often binds, the zebrafish model is highly valid model for studying drug effect in human. Solvents, oil dispersants, pesticides and metals are known to affect fish development deleteriously.

Teratogens

A teratogen is any medication, chemical, infectious disease, or environmental agent that might interfere with the normal development of a foetus and result in the loss of a pregnancy, a birth defect, or a pregnancy complication. There are a variety of teratogens that are relatively common. Some examples are listed, but there are other teratogens which are not on this list.

Recreational and Social teratogens

Two thirds of all infant mortalities are due to alterations in foetal development or development during the first year of life. Often times, recreational drugs significantly reduce foetal and post natal growth and are known to significantly increase infant mortality, such as alcohol (Gilbert, 1997; O'Rahilly, 1992), cocaine (Schardein, 1993), cigarettes (Klaassen, 1996). These teratogens usually

disrupt foetal development before the mother knows she is pregnant and has a chance to change her lifestyle.

Pharmaceutical teratogens

Most embryonic organs and the central nervous system are extremely sensitive to the teratogenic affects of pharmaceuticals in the early development. Often times these drugs causes embryonic malformations before the woman knows she is pregnant. Thalidomide (Schardein, 1993), Diethylstilbestrol (Schardein, 1993), Retinoic Acid (Schardein, 1993), Valproic Acid (Smith, 1982; Schardein, 1993), Warfarin (Smith, 1982; Schardein, 1993), chemotherapy (Brent, 1986), lithium (Gilbert, 2003; Stachel et al., 2003), nicotinic acid (Johnson and McColl, 1995) etc are some examples of Pharmaceutical teratogens.

Industrial and Environmental teratogens

In the modern era where rapidly growing industries are fulfilling the needs of growing population and has great economic importance, on the other hand these industries are releasing a huge amount of waste products in the environment. These waste products are not only polluting the environment but also leave severe detrimental effects on the quality of life. Among these a lot of waste products have teratogenic affects on the population such as organic solvents, chemicals, arsenic (Wlodarczyk et al., 1996), cadmium and lead (Weiss et al., 1986), anaesthetic gases, organic mercury(Harada, 1986) etc.

Agricultural teratogens

Studies have determined that insecticides (organochlorine insecticides) may interfere with fertility and reproduction by mimicking estrogen-like compounds. In some avian species, steroid metabolism is altered, making it impossible to transport calcium to the developing egg shell. Insecticides have also been found to accumulate and concentrate in the yolk sac of developing fish. Rodent studies of DDT showed reduced testicular size in males and estrogenic effects in female rats. Other teratogenic insecticides include dieldrin (reduction in fertility, increased mortality, delayed ossification and increased supernumerary ribs) and Kepone (reduction in sperm count and reduced motility).Herbicides, 2, 3, 7, 8-tetrachloro-dibenzo-*p*-dioxin (TCDD) were used in 2, 4, 5-T teratological studies resulting in cleft palate and congenital renal abnormalities.

Metabolic and Infectious Diseases

The TORCH complex is a group of similar malformations induced by microbial teratogens. These microbes affect 1-5% of all live births and are among the leading causes of neonatal morbidity and mortality. These organisms are Syphilis, Cytomegalovirus, rubella (South et al., 1986), cytomegalovirus (Reynolds et al., 1986), genital herpes, toxoplasmosis (Larsen, 1986), Fifth disease, and

chickenpox. Some metabolic disorders also induce malformations in the embryos e.g. Diabetes, hyperthermia (high fever). General symptoms include premature birth, growth retardation, neurological abnormalities, and damage of the eye, liver, heart and ear as well as bone lesions. Microcephaly, hydrocephaly, seizures and psychomotor retardation accompany these malformations.

On the basis of study documentation in animals and human and risk assessment to human teratogens are classified into different categories as shown in the table (A).

These categories are in agreement with FDA regulations as described in Millstein (1980): Table (A)

Category A	No demonstrated risk; possibility of foetal harm is remote.
Category B	Either: no evidence of foetal risk in extrapolation from animal data plus no information from human studies, or: evidence of adverse effects in animal studies that could not be confirmed in human studies.
Category C	Either: evidence of adverse effects in animal studies combined with no studies in humans, or: studies in animals and humans are not available. Drugs only given to pregnant women when the possible benefit outweighs the risks.
Category D	Positive evidence of foetal risk. Drugs may be given to pregnant women when the benefits are acceptable despite the risk.
Category E	Known not to affect animal reproduction but no human data.
Category X	Animal or human studies or human experience have revealed foetal abnormalities. Risk of use in pregnant women outweighs any possible benefits.

Model substances for the experiment

Retinoic acid (RA)

RA is the acidified form of Vitamin A. It is a drug commonly used to treat acne vulgaris and keratosis pilaris. In human foetus 13-cis-retinoic acid results in a characteristic pattern of anomalies, including absent or defective ears, absent or small jaws, cleft palate, aortic arch abnormalities, and abnormalities of the central nervous system. Similar anomalies are observed in other mammals. In mice, for example, embryonic exposure to retinoic acid results in axial truncation and causes a dramatic reduction in the sizes of the first and second pharyngeal arches,

which normally form the jaw, ear, and other facial bones (Gilbert, 2003; Ligas, 2000).

RA disrupts development by altering the expression of Hox genes, causing the re-specification of the anterior–posterior axis and inhibition of neural crest cell migration from the cranial region of the neural tube (Gilbert, 2003). RA cannot bind directly to genes, so in order to affect gene expression, the RA molecule needs to bind to specific RA receptors (RAR). After binding, the receptor becomes an active transcription factor. The RA-bound RARs have at least two modes of action, one of which is to bind to their DNA enhancer sequences and activate particular genes that are not usually activated in these cells. These genes include certain homeotic genes that specify the anterior-posterior position along the body axis. In this way, they can cause homeotic transformations, generally converting anterior structures into more posterior structures (Gilbert, 2003). In the embryo, there is a gradient of retinoic acid from the anterior end to the posterior end. Excess RA results in a posterior region having a higher than normal level of RA, and so more anterior Hox genes are expressed in typically posterior regions (Ligas, 2000).

Lithium

Lithium, in the form of lithium carbonate, is regularly prescribed to people with manic depressive (Bipolar) disorder. It takes the peaks and the valleys out of the emotional swings that a person with disease suffers, and is thought to do so through its control of receptors for the neurotransmitter glutamate. Since late 1800s embryologist have known from studies on non human embryos that lithium causes severe developmental defects. The attribute teratogenic effects are increased risk of heart defects especially Ebstein's anomaly which affects the tricuspid valve and seriously compromises heart ability to function effectively. Spina bifida and floppy infant syndrome are also reported defects.

In studies on non human embryos, the developmental defects caused by lithium are clear. As early as 1982 Herbst studied that echinoderm embryos exposed to lithium salts undergo exogastrulation. More recently it has been shown that both Xenopus and zebrafish embryos exposed to lithium during cleavage stages are dorsalized, showing severe defects in the posterior region (Gilbert, 2003; Stachel et al., 2003). These teratogenic defects are caused by lithium's inhibition of glycogen synthase kinase in the Wnt/β catenin or by depletion of inositol triphosphate in the PIP (phosphoinositol pathway) cycle as the dorsal-ventral axis is being established (Klein and Melton, 1996; Stachel et al., 1993; Baraban, 1994). In the zebra fish, later exposure (at the eleventh cleavage through gastrulation) causes anterior defects especially of the eyes, in some cases causing a lack of eyes altogether (Stachel et al., 1993).

Ethanol

Ethanol, widely consumed as a recreational drug, has long been strongly associated with teratogenesis as FAS (Jones et. al., 1973). FAS present up to one in three children of alcoholic mothers. FAS manifestations include growth

deficiency, CNS problems, characteristic facial features and organ malformations (Streissguth et al., 1995; Gilbert, 1997; Cartwright and Smith, 1995; Smith, 1997; Sulik et al., 1988). Each year, up to 40000 babies are born with some degree of alcoholic related damage (Sokol et. al., 2003).

Ethanol is able to permeate the placenta and enter foetal circulatory system, thereby causing developmental abnormalities. Ethanol impairs placental blood flow to the foetus by constricting blood vessels which induce hypoxia and foetal malnutrition. Ethanol rapidly crosses the placenta and blood brain barrier of the foetus. There are many proposed mechanism of action for ethanol such as altered neural crest cell migration, increased neural crest cell death or general cell death by superoxide radial lysis of cells or mitochondrial cell dysfunction. Ethanol may inhibit growth factor regulating cell proliferation and survival effects on Glial cells effects on development of neurotransmitter systems.

In zebrafish ethanol is responsible for abnormal migration of prechordal plate cells that ultimately causes cyclopia and other deformities (Blader and Strähle, 1998). The prechordal plate cell expressed genes like goosecoid and Islet 1, which control cell differentiation in the anterior region of the embryo (Blader and Strähle, 1998). Sulik (1988) studied that ethanol appears to cause abnormal cell death. Ethanol achieves apoptosis by activating the cell's self destruction machineries (Sulik et al., 1988). Therefore in addition to ectopic prechordal plates specific gene expression, ethanol induced apoptosis appears to contribute to the observed deformities in zebrafish embryos (Sulik et al., 1988).

6-Aminonicotinamide (6-AN)

6-Aminonicotinamide is a potent niacin antagonist (Johnson and McColl, 1995) having carcinostatic and teratogenic effects.6-AN is a reported teratogen in laboratory animals and induce cleft lip, stunted growth and hind limb defects in mice (Pinsky and Fraser ,1960) and similar malformations were observed in rabbit . Nicotinamide is transferred actively across the placenta (Hill and Longo, 1980; Kaminetzky et al., 1974) and into breast milk (Deodhar et al., 1964).Abnormal neural tube closure defects and other abnormalities were observed in chick when eggwhite was replaced with a solution containing 20 mg of nicotinic acid (Hansborough, 1974).

6-AN induce teratogenesis by inhibiting nicotinamide adenine dinucleotide dependent reactions during ATP synthesis. (Wilson et al., 1975; Dietrich et al., 1958).

Ochratoxin A (OA)

OA is an important food borne mycotoxin and is a potent teratogenic agent produced by several species of aspergillus and pencillium. OA is a reported teratogen in rats (Brown et. al.,1976; Mayura et al.,1982), mice (Hayes et al.,1974), Hamsters (Hood et al.,1976) and chicken (Gilani et al.,1975)

OA inhibits protein synthesis by competition with the amino acid phenylalanine (Phe) in the phenylalanyl-tRNA synthetase-catalyzed reaction (Bunge et al., 1978; Creppy et al., 1979).At post transcriptional level with OA having a direct effect on the translation step in the protein synthesis. This involves the competitive inhibition of phenylalanine-tRNA synthetase, so that aminoacylation and peptide elongation are stopped (Creppy et. al., 1979; WHO series28).

Manolova et al., (1990) described that one possible way of OA induction of teratogenesis is chromosomal aberrations by conducting positive *in vitro* experiment on human lymphocytes.

OA-DNA adducts formation in different parts of the foetus e.g. liver, kidney and other tissues, is a suggested mechanism of teratogenesis for OA by many scientist (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998).

Arsenic

Arsenic, a metal pollutant found naturally in groundwater and unnaturally in mine waste sites and agricultural runoff, has been considered toxic to humans for several millennia. Arsenic is teratogenic, and it has been shown to cross the mammalian placenta, affecting developing embryos whose mothers undergo exposure (Wlodarczyk et al., 1996).

Arsenic has been found to cause high mortality, miscarriage, stillbirth, developmental retardation and birth defects in humans (Shalat et al., 1996); neural tube, ocular and jaw defects (Stump et al., 1999) and behavioural retardation and delayed ear detachment (Rodriguez et al., 2002) in rodent embryos; and everted internal organs and small eyes in chicks (Shalat et al., 1996). Neural tube defects have been identified as a common defect among mammals, possibly reflecting the fact that arsenic accumulates in the neuroepithelium of developing fetuses (Shalat et al., 1996). Inorganic arsenic, of which sodium arsenate and arsenic trioxide are two kinds, may cause neural tube defects by repressing cell replication through microtubule organization or by inhibiting cell shape changes necessary to neural tube formation (Shalat et al., 1996). Wlodarczyk and co-workers (1996) suggest that arsenic damaging of DNA is responsible for inhibition of cell propagation, thus delaying and preventing a normal neural tube closure.

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Aim of the study

The aim of the present study was to assess the potential of the zebrafish as a laboratory animal model for teratogen testing. Potential human teratogens i.e. Retinoic Acid , Lithium Hydroxide, Ethanol, Arsenic Oxide, Ochratoxin A and 6–Aminonicotinamide were used on zebrafish embryos. Results of the study were compared with the results of other laboratory animals

RESEARCH PAPER

Teratology in zebrafish embryos: A tool for risk assessment

Nadeem Ali*, Gunnar Carlsson and Leif Norrgren

Department of Biomedical Sciences and Veterinary Public Health,Division of Pathology, Pharmacology and Toxicology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), P.O. Box 7028, SE- 750 07 Uppsala, Sweden
*Corresponding author email: chakrian2010@yahoo.co.uk

Abstract

Teratology, the study of abnormal prenatal development and congenital malformations induced by exogenous chemical and physical agents, is one of the debating areas of medical research in the quest for the eradication of preventable birth defects. Identification of agents with teratogenic potential from the huge amount of drugs that human being come into contact within their every day environment is crucial because they are compromising the quality of life for millions of people world wide. Since the thalidomide catastrophe in 1961 regulatory agencies have instigated requirements for new drugs to be thoroughly tested on animals prior to approval for marketing. Different laboratory animals mainly rat, mice, rabbit, primates are being used for chemicals teratogenicity tests but none of these laboratory animal models fulfil the criteria for ideal experimental model. In the recent times zebrafish has emerged as a prominent developmental study model.

Fertilized eggs of zebrafish were used to test teratogenicity of chemicals. Zebrafish embryos were cultured in 96 -well plates, with a range of different concentrations of the chemicals, a method described by Schulte and Nagel (1994). The following parameters of the development of *Danio rerio* were observed at every 24hpf until 144hpf: coagulation of eggs, hatching, movement, circulation, heart rate, pigmentation, development of eyes, fins and tail, and yolk sac oedema. Six chemicals from different teratogenic classes (retinoic acid, lithium hydroxide, Ochratoxin A, 6-aminonicotinamide, sodium arsenate and ethanol) were selected for the teratogenic testing in the zebrafish embryos. The developmental defects induced by these chemicals in zebrafish embryos were studied. The results were compared with other laboratory animal's available data about the substances. All chemicals except sodium arsenate induced severe developmental defects among zebrafish embryos.

Introduction

Teratology, the study of abnormal prenatal development and congenital malformations induced by exogenous chemical or physical agents, is a growing area of medical research in the quest for the eradication of preventable birth defects. Birth defects are known to occur in huge numbers roughly seven to ten percent of all children require extensive medical care to diagnose or treat a birth defect, this compromise the quality of life for millions of people world wide (Dicke, 1989). Identification of agents with teratogenic potential from the huge amount of drugs and chemicals that human being come into contact with in their everyday environment in crucial. It is estimated that 10% of all birth defects are caused by a prenatal exposure or teratogen (O'Rahilly, 1996). A good knowledge about these hazardous substances would enable medical professionals and would-be mothers to minimize foetal exposure to them. This will help in achieving the laudable goal of abolishing teratogen- induced malformations.

Since Thalidomide catastrophe in 1961 (Mc Bride, 1961; Lenz, 1966) regulatory agencies such as United States Food and Drug Administration (FDA) instigated requirements for new drugs to be thoroughly tested on animals prior to approval for marketing. Currently the burden of identifying of human teratogens has rested squarely on the shoulders of animal-based research. Animal-based studies of developmental toxicology provide the initial guideline whether a drug or chemical may present a teratogenic risk during pregnancy. The most prevalent species used are mice, rats, rabbits, hamsters and primates, but no one species fulfilling the ideal teratology animal criteria such as "producing large litters after a short gestation, in expansive maintenance and inability and unwillingness to 'bit, scratch, kick, howl, squeal'". Scientist are still exploring new alternative for teratogen testing. Recently zebrafish (*Danio rerio*) has emerged as a competitive laboratory animal model species and has been used as a model species for developmental biology.

The zebrafish (*Danio rerio*) (Hamilton-Buchanan, 1922; formally Brachydanio rerio) is a small cyprinid found in tributaries and branches of the Ganga river in South-East Asia (Eaton and Farley, 1974). This species measures 3 to 5 cm as an adult and thrives both in soft and hard water. At 26°C zebrafish grow quickly and reaches maturity within 3 months (Skidmore, 1967).

Zebrafish is easily obtainable, inexpensive and readily maintainable. Under appropriate conditions it provides a large number of non-adherent and transparent eggs i.e. approximately 50 to 200 eggs per female per day (Laale, 1977). Zebrafish embryonic development has been described in numerous studies and has been used as model in numerous fields of molecular genetics, vertebrate developmental biology, neurobiology and transgenic research (Roosen-Runge, 1938; Thomas and Waterman, 1978; Kimmel et at., 1988; Hisaoka and Battle, 1958; Laale, et al., 1977; Sander and Baumann, 1983; Nagel, 1988; Kimmel et al., 1995; Nusslein-Volhard, 1994; Westerfield, 1995; Lele and Krone, 1996; Goolish et al., 1999; Wixon, 2000). From the last decade of 20th century and start

of 21st century studies showed that the zebrafish has become one of the most important models for vertebrate developmental biology (Ekker and Akimenko, 1991; Nusslen- Volhard, 1994; Westerfield, 1995).

There are numerous advantages for the use of zebrafish as a toxicological model species (Spitsbergen and Kent, 2003; Teraoka et al., 2003) as well as for other disciplines. This is evident by the number of publications about zebrafish studies in the recent past. In early 1990s there was less than one hundred zebrafish publications annually submitted. This rose to around about thousands at the turn of the century and now the average is around 3500 per year.

In contrast to other vertebrate models the zebrafish model has great benefits with regards to their sizes, husbandry and early morphology. Zebrafish has high fecundity, small size (3 to 5cm), short generation time, rapid development, external fertilization, translucent embryos and is easy to maintain at laboratory level (Laale, 1997). This greatly reduces housing space and husbandry cost. Zebrafish has also been utilized as a laboratory species for quite some time, so the optimum breeding and maintenance conditions have been well documented (Westerfield, 1995). In contrast to larger laboratory species the minute size of larval and adult zebrafish minimizes costs through low quantities of dosing solutions (Only nanogram quantities of experimental chemicals, drugs and pollutants) and thereby creates limit volumes of waste for disposal. Small size of zebrafish minimize the quantity of labware and chemicals both for treating and maintaining live fish and for performing various assay (low quantities of reagents and histological assessments, small amount of embedding materials and microscope slides) (Hill et al.,2002). Small embryos allow reasonable sample size and several experimental replicates at one time. The zebrafish embryos can grow, develop and survive for several days in a single well of a 384-well plate and 96-well plate through the absorption of yolk and can be visually assessed for malformations (Nagel, 1993; Mac Rae and Peterson, 2003). The zebrafish embryo is completely transparent so it facilitates visual multi-parameter observation and analysis at a time. In one screen the cardiovascular system, central nervous system and skin can be assessed with a dissection microscope (Peterson et al., 2000). Zebrafish embryo's entire body plan develop by in 24 hours and all the precursor cell and the tissues of the brain, eyes, heart and musculature can be easily visualized using light microscope. The zebrafish completes embryogenesis in the first 72 hours and most of the internal organs including the cardiovascular system, gut, liver, and kidney develop rapidly in the first 24 to 48 hours (Stainier and Fishman, 1994). Unlike rodents, embryological development can be continually followed in live individuals rather than in harvested embryos and foetuses. In addition, zebrafish embryos that are malformed, missing organs, or displaying organ dysfunction, can usually survive substantially past the time in which those organs start to function in healthy individuals. For example, mutant zebrafish such as silent heart, still heart, and slow mo (Chen et al., 1996), and toxicant-exposed embryos with heart abnormalities (Antkiewicz et al., in press; Incardona et al., 2004) survive well beyond 24hpf when the heart normally begins to beat (Kimmel et al., 1995). This is in contrast to rodent embryos with malformed hearts that tend to die in utero. An important advantage of zebrafish

model is that the morphological and molecular basis of tissues and organ development are in general either identical or similar to other vertebrates including men (Chen and Fishman, 1996; Granato and Nusslein-volhard, 1996). There is extensive similarity between the zebrafish and human genomes so many human developmental and disease genes have counterparts in the zebrafish. The zebrafish genome is 1700 million base pairs in length, about half the size of the human genome. Most human genes have homologues to zebrafish and the functional domain of the proteins such as ATP binding domain of kinases are almost 100% identical between homologous genes, although the similarity over the entire protein is about 60 % (Langheinrich, 2003).

In the present study, the teratogenicity of six chemicals was studied by using fertilized eggs of the zebrafish (*Danio rerio*). These chemicals were selected from different categories of potential human teratogens with different mechanisms of action to induce teratogenesis. Ethanol, widely consumed as a recreational drug, has long been strongly associated with teratogenesis as foetal alcohol syndrome (FAS) (Jones et. al., 1973). FAS is present up to one in three children of alcoholic mothers. FAS manifestations include growth deficiency, CNS problems, characteristic facial features and organ malformations (Streissguth et al., 1995; Gilbert, 1997; Cartwright and Smith, 1995; Smith, 1997; Sulik et al., 1988). To induce malformations ethanol alters the mechanism of cell migration in the developing foetus (Blader and Strähle, 1998) and in some times cell death (Sulik et al., 1988). Retinoic acid (RA) is the acidified form of Vitamin A. It is a drug commonly used to treat acne vulgaris and keratosis pilaris. In human foetus 13-cis-retinoic acid results in a characteristic pattern of anomalies, including absent or defective ears, absent or small jaws, cleft palate, aortic arch abnormalities, and abnormalities of the central nervous system. RA disrupts development by altering the expression of Hox genes, causing the re-specification of the anterior–posterior axis and inhibition of neural crest cell migration from the cranial region of the neural tube (Gilbert, 2003). Lithium, medicine for manic depressive (Bipolar) disorder, causes severe developmental defects the attribute teratogenic effects are increased risk of heart defects especially an Ebstein's anomaly which affects the tricuspid valve and seriously compromises heart ability to function effectively. Spina bifida and floppy infant syndrome are also reported defects. These teratogenic defects are caused by lithium's inhibition of glycogen synthase kinase in the Wnt/β catenin and by depletion of inositol triphosphate in the PIP (phosphoinositol pathway) cycle as the dorsal-ventral axis is being established (Klein and Melton, 1996; Stachel et al., 1993; Baraban, 1994). Arsenic, a metal pollutant, cause high mortality, miscarriage, stillbirth, developmental retardation and birth defects in humans that include neural tube, ocular and jaw defects (Stump et al., 1999; Shalat et al*.,* 1996). Wlodarczyk et. al. (1996) suggested that arsenic damaging of DNA is responsible for inhibition of cell propagation, thus delaying and preventing a normal neural tube closure. Ochratoxin A (OA) ,a food borne mycotoxin, is a reported teratogen in rats (Brown et. al.,1976; Mayura et al.,1982), mice (Hayes et al.,1974), Hamsters (Hood et. al.,1976) and chicken (Gilani et al.,1975). The mechanism of teratogenesis of OA is to inhibit protein synthesis by competition with the amino acid phenylalanine (Phe) in the phenylalanyl-tRNA synthetase-catalyzed reaction (Bunge et al., 1978; Creppy et

al., 1979). 6 -Aminonicotinamide is a potent niacin antagonist (Johnson and McColl, 1995) having carcinostatic and teratogenic effects.6-AN is a reported teratogen in laboratory animals and induce cleft lip, stunted growth and hind limb defects in mice (Pinsky and Fraser, 1960). 6-AN induce teratogenesis by inhibiting nicotinamide adenine dinucleotide dependent reactions during ATP synthesis (Wilson et al., 1975).

The aim of the present study was to observe the potential of zebrafish as a laboratory animal species for teratogen testing by using the above mentioned human potential teratogens. Results of the study were compared with the results of other laboratory animal species.

Materials and method

Test material

Six different chemicals with known potential human teratogenicity were used for the study, these chemical were bought from the respective companies as shown in the table (a). Experiments were performed at the Department of Biomedical Sciences and Veterinary Public Health, Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Chemical	**Trade name**	**Company**	**Chemical formula**	**Molecular weight**
Retinoic acid	Tretinoin®	F. Hoffmann-La Roche Ltd, Switzerland	$C_{20}H_{28}O_2$	300.42
Lithium hydroxide	LiOH 98%®	E.Merck,Germany	LiOH	23.95
Ochratoxin A	Ochratoxin A®	Fluka AG,chem. Switzerland	$C_{20}H_{18}ClNO_6$	403.81
Ethanol 96%			C_2H_5OH	46.07
6-Amino nicotinamide	6-Aminonicotin amide, purum, ≥99.0% (NT) ®	Fluka AG, chem. Switzerland	$C_6\ H_7\ N_3\ O$	137.14
Arsenic	Sodium arsenate dibasic hepta hydrate ≥98.0%	Sigma-Aldrich	$Na_2HAsO_4 \cdot 7H_2O$	312.01

Animals

Fertilized eggs of zebrafish (Danio rerio; cyprinidae, Hamilton-buchanan 1822) were used. Adult zebrafish (*Danio rerio*), originally bought from a local importer (akvarium kalhäll, Jakobsberg, Sweden), were adapted in the fish laboratory at the Department of Biomedical Sciences and Veterinary Public Health, Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural

Sciences, Uppsala , Sweden. The fish were kept in the laboratory at a temperature of 26±1°C and 12:12 hours day/night regimen. The fish were fed twice a day with sera micron (Sera ®, Germany), Artemia nauplii and powdered freeze-dried red grubs (Nutrafin ®, Taiwan).). The fish were kept in constantly aerated, standardised water ($CaCl_2x2H_2O$ (117.6 mg/litre), $MgSO_4x7H_2O$ (49.3 mg/litre), $NaHCO_3$ (25.9 mg/litre) and KCl (2.3 mg/litre)) (ISO 7346-1, 1996).

Test design

On the day before the experiment, phenotypic zebrafish females (5-10) and males (10-20) from the main aquarium were transferred to a breeding funnel with 15L standardised water. A net cage with 4mm holes was kept in the breeding funnel to keep adult zebrafish away from the eggs. In the morning, once the light was turned on, the fish begun to mate. The eggs were collected after 30 minutes from the bottom tap of the breeding funnel and rinsed twice in standardised water .Fertilized eggs were selected with a dissecting microscope. After egg collection, the adult zebrafish were returned to the main aquarium.

Exposure and embryo developmental test

Fertilized eggs that had reached the four cell stage were exposed to a range of concentrations of potential human teratogenic substances.

Retinoic acid (10^{-9} M, 10^{-8}M, $10^{-7.8}$M, $10^{-7.6}$M, $10^{-7.4}$M, $10^{-7.2}$M, 10^{-7}M)

Lithium hydroxide ($1x10^{-2}$M,$2x10^{-2}$M, $3x10^{-2}$M, $4x10^{-2}$M, $8x10^{-2}$M, $12x10^{-2}$M, $16x10^{-2}$M)

Ochratoxin A ($0.5x10^{-7}$M, $1x10^{-7}$M, $2x10^{-7}$M, $3x10^{-7}$M, $6x10^{-7}$M, $12x10^{-7}$M, $25x10^{-7}$M)

6Aminonicotinamide ($0.5x10^{-6}$M, $1x10^{-6}$M, $3.5x10^{-6}$M, $7x10^{-6}$M, $14x10^{-6}$M, $22x10^{-6}$M, $29x10^{-6}$M)

Sodium arsenate ($1.5x10^{-3}$M,$3x10^{-3}$M,$6x10^{-3}$M,$9x10^{-3}$M,$12x10^{-3}$M,$15x10^{-3}$M)

Ethanol (0.5%, 1%, 1.25%, 1.5%, 1.75%, 2%, 2.5%)

This method was described by Schulte and Nagel (1994). Selected fertilized eggs that had reached the four cell stage were transferred into the individual u–shaped wells of 96 well styrene plate (costar, coring incorporated, USA) by the use of a pipette filled with rearing media. DMSO was used as a solvent for RA and OA; all other substances were water soluble. Each well contained 250µl rearing media which was not changed during the study. 24 eggs were used per concentration group and the study was done twice for each substance. The styrene plates were covered with plastic sheets to avoid evaporation and were incubated at a constant temperature of 26ºC. Embryos were examined under dissecting microscope after

every 24 hours for 6 days, after which the embryos were euthanized with MS-222 (200mg/liter) solution. Microscopic examination was performed by use of a light source equipped with a fiber optic cable to prevent temperature stress of the embryos.

Statistics

Heart rate was analysed statistically using one way ANOVA followed by Dunnett's post hoc test. Hatching and coagulation were analysed by fisher exact test. All other ordinal parameters were analysed by using Kruskal-Wallis test followed by Mann-Whitney test, where each group was compared with respective control group. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were calculated. The significance level was set at 0.95 ($P<0.05$). The analyses were made by using Statview 5.0.1 (SAS institute Inc.) and MINITAB release 14 (Minitab Inc.). Sidan:32.

End points of the study

Endpoints studied at different stages of the development in zebrafish embryos;

Endpoint	Description	24hpf	48hpf	72hpf	96hpf	120hpf	144hpf
Coagulation	Embryo coagulation 0=no coagulation 1=coagulated	+	+	+	+	+	+
Movement	Movement within 30 seconds 1=normal movement. 2=sluggish movement 3=no movement at all	+	+	+	+	+	+
Heart rate	Beats/minutes		+				
Circulation	Visible blood flow in tail region 1=normal flow 2=affected circulation 3=no circulation		+	+	+	+	+
Oedema	Oedema around the body 0=no oedema 1=slight oedema in heart region 2=moderate oedema in heart rate 3=severe oedema in heart region 4=severe oedema involving whole body.	+	+	+	+	+	+
Eyes	Size and colour of eyes 1=normal size and colour 2=relatively small size 3=very small or no eyes at all.	+	+	+	+	+	+
Tail extension	Length of tail, separation from yolk sac 1=normal tail 2=relatively short tail 3= very short tail and attached to yolk sac	+	+	+	+	+	+
Pigmentation	Pigmentation in the body 1=normal pigmentation 2=slight pigmentation 3=no pigmentation		+	+	+	+	+
Hatching	Embryo hatching 0=not hatched 1=hatched			+	+	+	+
Fins	Length of fins 1=normal length 2=small fins 3=no fin development				+	+	+

Results

Embryos were continuously monitored till 144hpf. Readings of the selected end points were taken after every 24 hours for 6 days. For the statistical analysis data taken by 48hpf and 144hpf were selected because most of the studied end points were developed by 48hpf and heart rate was easy to count, and 144hpf was the study termination point.

Retinoic acid

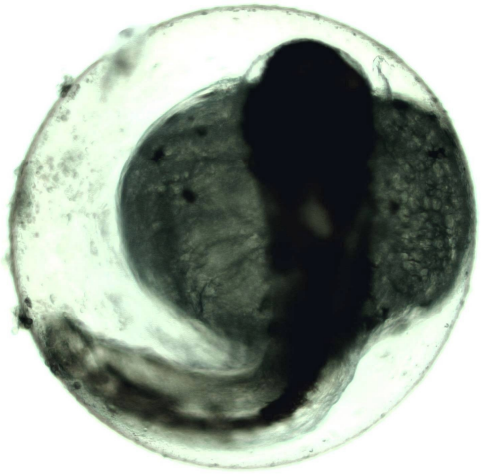
Zebrafish embryos treated with RA at concentrations of 10^{-8}M and 10^{-9} M presented no apparent developmental abnormalities. By approximately 48hpf, the embryos displayed no evidence of truncation and had apparently normal development. Normal development was observed 2 to 3 days after treatments. By which most of embryos had hatched. No difference in heart rate was observed with respect to control group (Table I). The interior and posterior regions of embryos developed similarly to that of control. These embryos survived until euthanized.

The embryos treated with higher concentrations of RA$10^{-7.8}$M and $10^{-7.6}$M were severely affected. These embryos presented truncation of the interior and posterior axis by 48hpf. Heart rate of these embryos was significantly different from the control group. The yolk sacs were on average noticeably larger than those of the control embryos. Embryos treated with $10^{-7.4}$M, $10^{-7.2}$M, 10^{-7}M had severe under development and larger oedema, no movement, no circulation, small eyes, short tail. Most of the embryos treated with these concentrations of RA died by the 3rd day after treatment (Table I).

Table text
(= significant different)*
(- = no statistically significant data)
(HR = Heart rate beats/minute)
(HPF = hours post fertilization)
(Median values for all ordinal parameters and mean values for heart rate)
(Statistical analysis was done for the results when at least 5 embryos were alive out of 24 individuals)

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
Retinoic acid	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	138.5
10^{-9} M		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	134.5
10^{-8}M		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	135.5
$10^{-7.8}$M		0.0	1.0	1.0	1.0*	1.0	1.0	1.0	0.0	125.5*
$10^{-7.6}$M		0.0	1.0	2.0*	1.0*	1.0	1.0	1.0	0.0	117.5*
$10^{-7.4}$M		0.0	2.5*	3.0*	3.0*	2.0*	2.0*	2.0*	0.0	-
$10^{-7.2}$M		0.0	3.0*	3.0*	3.0*	3.0*	2.0*	2.0*	0.0	-
10^{-7}M		0.0	3.0*	3.0*	4.0*	3.0*	2.0*	2.0*	0.0	-
Concentration	**Time**	**Coagulation**	**Movement**	**Circulation**	**Oedema**	**Eyes**	**Tail**	**Pigmentation**	**Hatching**	**Fins**
Retinoic acid	144hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
10^{-9} M		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
10^{-8}M		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$10^{-7.8}$M		0.5*	2.5*	1.0	2.0*	1.5*	1.0	1.0	1.0	1.0
$10^{-7.6}$M		0.5*	3.0*	2.5*	2.5*	2.0*	2.0*	1.0	1.0	1.5*
$10^{-7.4}$M		0.5*	3.0*	3.0*	3.0*	3.0*	2.0*	2.0*	1.0	3.0*
$10^{-7.2}$M		1.0*	3.0*	3.0*	3.0*	3.0*	2.0*	2.0*	1.0	3.0*
10^{-7}M		1.0*	-	-	-	-	-	-	-	-

Table (I); Statistical analysed studied end point results for RA by 48hfp and 144hpf.



A

B

Figure (A) $10^{-7.4}$ M RA treated embryos by 144hpf. No development of eyes, very short tail, yolk sac oedema and not hatched (B) $10^{-7.8}$M RA treated embryos by 144hpf. Microphthalmia and yolk sac oedema.

Lithium hydroxide

Zebrafish embryos treated with LiOH at the concentration ($1x10^{-2}M$, $2x10^{-2}M$, $3x10^{-2}M$, $4x10^{-2}M$, $8x10^{-2}M$, $12x10^{-2}M$ and $16x10^{-2}M$) showed normal development and presented no apparent developmental abnormalities by 48hpf. By approximately 144hpf, embryos treated with $1x10^{-2}M$, $2x10^{-2}M$ and $3x10^{-2}M$ continued normal development as to that of control group. Most of the embryos treated with $4x10^{-2}M$,$8x10^{-2}M$ and$12x10^{-2}M$ coagulated by day six and no coagulated embryos showed affected movement, no circulation and severe truncation of interior and posterior axis. Most of the embryos exhibited scoliosis. Coagulation rate was less among the embryos treated with $16x10^{-2}M$ but they had developed severe developmental abnormalities by 144hpf i.e. no movement, no circulation, small eyes, short tail, under developed fins and scoliosis (Table II).

Figure. 12x10-2M Lithium hydroxide treated embryo after 144hfp. Microphthalmia, yolk sac oedema and relatively short tail.

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
Lithium hydroxide	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	136.5
$1x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	134.5
$2x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	135.5
$3x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	133.5
$4x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	132.5
$8x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	135.5
$12x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	132.5
$16x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	132
Concentration	**Time**	**Coagulation**	**Movement**	**Circulation**	**Oedema**	**Eyes**	**Tail**	**Pigmentation**	**Hatching**	**Fins**
Lithium hydroxide	144hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$1x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$2x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$3x10^{-2}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$4x10^{-2}M$		0.0	3.0*	3.0*	2.5*	1.0	1.0	1.0	1.0	2.5*
$8x10^{-2}M$		1.0*	3.0*	3.0*	3.0*	1.0	1.0	1.0	1.0	3.0*
$12x10^{-2}M$		1.0*	3.0*	3.0*	3.0*	2.0*	2.0*	1.0	1.0	3.0*
$16x10^{-2}M$		0.0	3.0*	2.5*	3.0*	2.0*	2.0*	1.0	1.0	3.0*

Table (II); Statistical analysed studied end point results for Lithium hydroxide by 48hfp and 144hpf.

Ethanol

Development was normal among the zebrafish embryos treated with 0.5 %, 1 % and 1.25 % ethanol by 48hpf (Table III). Affected circulation and moderate yolk sac oedema was observed among the zebrafish embryos treated with 1.75% and 1.5% by 48hpf. Zebrafish embryos treated with 2% and 2.5% ethanol develop severe developmental abnormalities i.e., lower heart rate, affected circulation, moderate to severe yolk sac oedema, microphthalmia to anophthalmia and affected tail development by 48hpf (Table III).

By approximately 144hpf embryos treated with 0.5% and 1% ethanol developed normally and presented no developmental abnormalities. 60% of the embryos treated with 1.25% ethanol coagulated and no coagulated embryos showed only slight yolk sac oedema and others parameters were normal. 70-80% of the embryos treated with 1.5% and 1.75% ethanol were coagulated and no coagulated embryos showed severe oedema, lack of movement, microphthalmia and under developed fins. All embryos treated with 2 %and 2.5% ethanol were coagulated by the sixth day (table III).

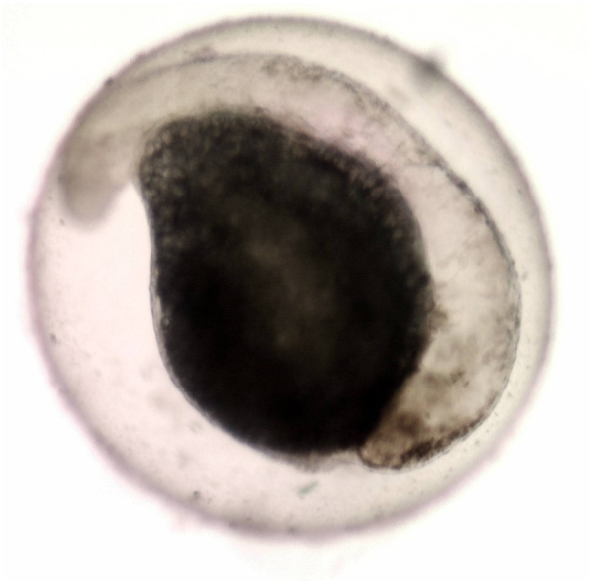


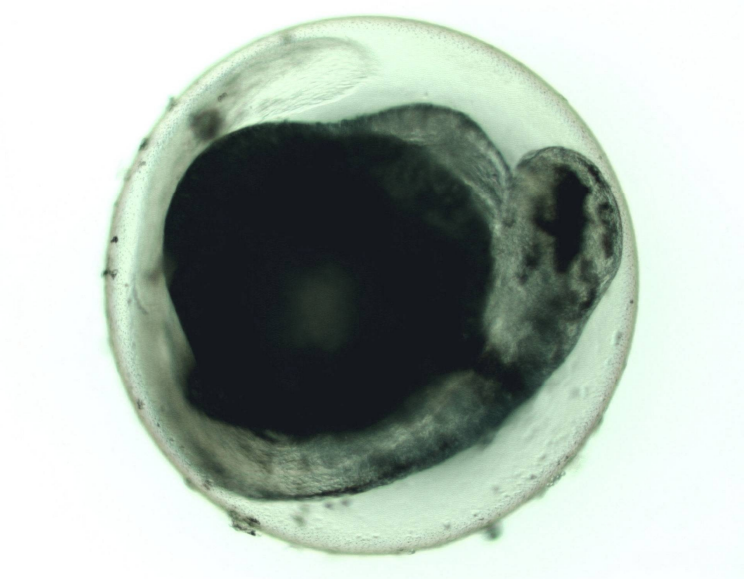
Figure. 2.5% Ethanol treated embryo after 72hfp. Large yolk sac oedema and no development of eyes and tail.

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
Ethanol	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	140.5
0.5%		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	137.5
1.0%		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	138.0
1.25%		0.0	1.0	1.0	1.0*	1.0	1.0	1.0	0.0	137.5
1.5%		0.0	1.0	2.0*	2.0*	1.0	1.0	1.0	0.0	136.5
1.75%		0.0	1.0	1.5*	2.0*	1.0	1.0	1.0	0.0	124.5*
2.0%		0.0	2.0*	2.0*	2.0*	1.5*	1.5*	1.0	0.0	118.5*
2.5%		0.5*	3.0*	3.0*	4.0*	3.0*	3.0*	1.5*	0.0	-
Concentration	**Time**	**Coagulation**	**Movement**	**Circulation**	**Oedema**	**Eyes**	**Tail**	**Pigmentation**	**Hatching**	**Fins**
Ethanol	144hpf									
Control		0.0	1.0	1.0	0	1.0	1.0	1.0	1.0	1.0
0.5%		0.0	1.0	1.0	0	1.0	1.0	1.0	1.0	1.0
1.0%		0.0	1.0	1.0	0	1.0	1.0	1.0	1.0	1.0
1.25%		0.5*	1.0	1.0	1.0*	1.0	1.0	1.0	1.0	1.0
1.5%		1.0*	1.0	1.0	2.0*	2.0*	1.0	1.0	1.0	2.0*
1.75%		1.0*	3.0*	3.0*	2.5*	3.0*	2.0*	2.0*	0.5*	3.0*
2.0%		1.0*	-	-	-	-	-	-	-	-
2.5%		1.0*	-	-	-	-	-	-	-	-

Table (III); Statistical analysed studied end point results for ethanol by 48hfp and 144hpf.

6-Aminonicotinamide

By 48hpf zebrafish embryos cultured in higher concentrations of 6-AN ($7x10^{-6}$M, $14x10^{-6}$M, $22x10^{-6}$M and $29x10^{-6}$M) showed developmental abnormalities, i.e. moderate yolk sac oedema , lack of movement, affected eyes. Lower pigmentation was observed only in $29x10^{-6}$M treated embryos (Table IV). Some embryos treated with $3.5x10^{-6}$M lacked spontaneous movement and developed slight oedema around the yolk sac by 48hpf. Normal development was observed among the zebrafish embryos treated with $0.5x10^{-6}$M and $1x10^{-6}$M treatment of 6-AN by 48hpf and 144hpf. Embryos treated with $3.5x10^{-6}$M, $7x10^{-6}$M, $14x10^{-6}$M, $22x10^{-6}$M and $29x10^{-6}$M showed severe developmental abnormalities by 144hpf, i.e. no movement, microphthalmia, severe yolk sac oedema and 15-20% coagulation rates in all groups and lower pigmentation only in $29x10^{-6}$M treated embryos. Hatching was also affected and embryos treated with $14x10^{-6}$M, $22x10^{-6}$M and $29x10^{-6}$M showed lack of hatching (Table IV).



A

B

Figure (A) 22x10-6M 6-AMN treated embryo after 144hpf. No hatching and no development of eyes (B) 3.5x10-6M 6-AMN treated embryo after 144hpf. Yolk sac oedema, relatively short tail and eyes as compare to control embryos.

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
6-Aminonictinamide	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	140.5
$0.5x10^{-6}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	137.5
$1x10^{-6}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	139.0
$3.5x10^{-6}M$		0.0	1.5*	1.0	1.0*	1.0	1.0	1.0	0.0	137..0
$7x10^{-6}M$		0.0	2.0*	1.0	2.0*	1.5*	1.0	1.0	0.0	132.5*
$14x10^{-6}M$		0.0	3.0*	1.0	2.0*	2.0*	1.0	1.0	0.0	124.5*
$22x10^{-6}M$		0.0	3.0*	2.0*	2.0*	2.0*	1.0	1.0	0.0	120.5*
$29x10^{-6}M$		0.0	3.0*	2.0*	2.0*	2.0*	2.0*	2.0*	0.0	115.5*

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	Fins
6-Aminonictinamide	144hpf									
Control		0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$0.5x10^{-6}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$1x10^{-6}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$3.5x10^{-6}M$		0.5*	2.0*	2.0*	2.5*	2.0*	2.0*	1.0	1.0	2.5*
$7x10^{-6}M$		0.5*	3.0*	3.0*	3.0*	2.0*	2.0*	1.0	1.0	3.0*
$14x10^{-6}M$		0.5*	3.0*	3.0*	2.0*	2.0*	2.0*	1.0	0.0*	3.0*
$22x10^{-6}M$		0.0	3.0*	3.0*	2.0*	3.0*	2.0*	1.0	0.0*	3.0*
$29x10^{-6}M$		0.0	3.0*	3.0*	3.0*	3.0*	2.0*	2.0*	0.0*	3.0*

Table (IV); Statistical analysed studied end point results for 6-AN by 48hfp and 144hpf.

Ochratoxin A

At higher concentrations of OA ($6x10^{-7}$M, $12x10^{-7}$M and $25x10^{-7}$M) by 48hpf embryos showed developmental abnormalities, i.e. oedema, affected circulation and significantly lower heart rate in $12x10^{-7}$M and $25x10^{-7}$M treated embryos. Embryos treated with lower concentrations $0.5x10^{-7}$M, $1x10^{-7}$M, $2x10^{-7}$M and $3x10^{-7}$M continued to develop normal as that of the control group and no abnormalities were observed 48hpf (Table V).

By 144hpf embryos treated with $0.5x10^{-7}$M and $1x10^{-7}$M concentration of OA exhibited no developmental abnormalities and continued normal development. All of the embryos treated with $12x10^{-7}$M and $25x10^{-7}$M were coagulated. 60% coagulation was observed among the zebrafish embryos treated with $6x10^{-7}$M and no coagulated embryos exhibited developmental abnormalities, i.e. lack of movement, severe yolk sac oedema, affected circulation, under developed fins and affected eyes. Embryos treated with $2x10^{-7}$M and $3x10^{-7}$M showed some developmental abnormalities, i.e. slight oedema and coagulation. In $3x10^{-7}$M treated embryos observed developmental abnormalities were not significantly different from the control embryos (Table V).

A

B

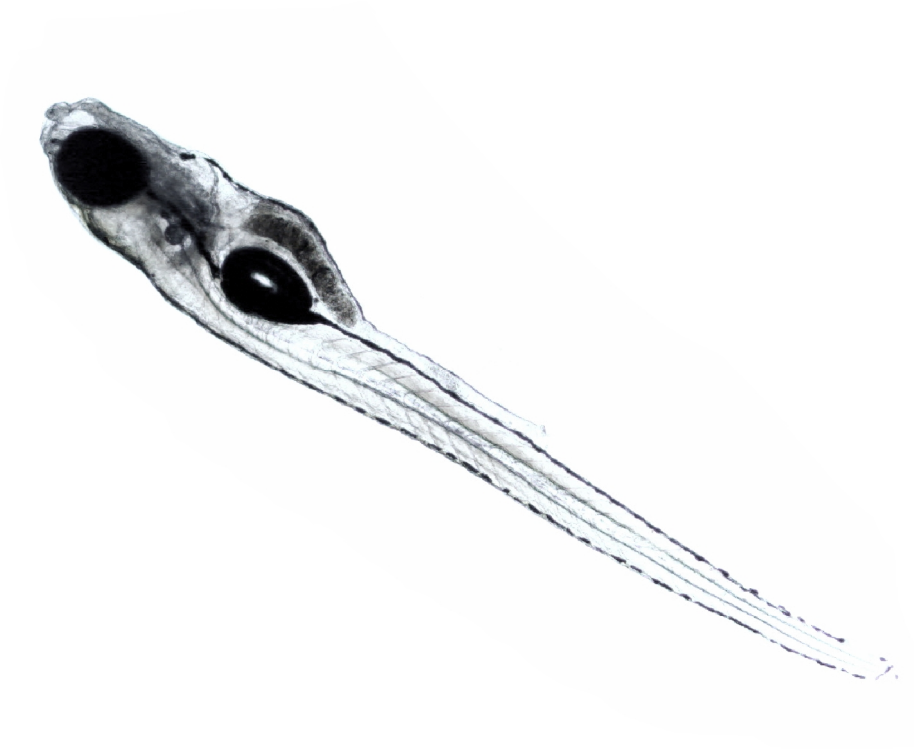
Figure (A & B) 6x10-7M Ochratoxin A treated embryo after 144hpf. Development of large yolk sac oedema.

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
Ochratoxin A	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	141.0
$0.5x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	144.5
$1x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	142.5
$2x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	135.0
$3x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	138.5
$6x10^{-7}M$		0.0	1.0	1.5*	0.0	1.0	1.0	1.0	0.0	136.5
$12x10^{-7}M$		0.0	1.0	2.5*	1.0*	1.0	1.0	1.0	0.0	130.0*
$25x10^{-7}M$		0.0	1.0	3.0*	2.0*	1.0	1.0	2.0*	0.0	125.5*
Concentration	**Time**	**Coagulation**	**Movement**	**Circulation**	**Oedema**	**Eyes**	**Tail**	**Pigmentation**	**Hatching**	**Fins**
Ochratoxin A	144hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$0.5x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$1x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$2x10^{-7}M$		0.5*	1.0	1.5*	1.5*	1.0	1.0	1.0	1.0	1.0
$3x10^{-7}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$6x10^{-7}M$		1.0*	3.0*	3.0*	3.0*	1.5*	1.0	1.0	1.0	2.0*
$12x10^{-7}M$		1.0*	-	-	-	-	-	-	-	-
$25x10^{-7}M$		1.0*	-	-	-	-	-	-	-	-

Table (V); Statistical analysed studied end point results for OTA by 48hfp and 144hpf

Sodium arsenate

Zebrafish embryos provide no evidence of phenotypic abnormal development when cultured in sodium arsenate rearing media even as high as $16x10^{-3}$M solution by 48hpf. After day six of sodium arsenate treatment all exposed embryos developed normally as that of control group (Table VI).



12x10-3M Sodium arsenate treated embryo after 144hpf. No developmental abnormalities, all studied parameters are normal

Concentration	Time	Coagulation	Movement	Circulation	Oedema	Eyes	Tail	Pigmentation	Hatching	HR (mean)
Sodium arsenate	48hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	141.5
$1.5x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	138.5
$3x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	140.0
$6x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	137.5
$9x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	139.5
$12x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	138.5
$15x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	0.0	137.0
Concentration	**Time**	**Coagulation**	**Movement**	**Circulation**	**Oedema**	**Eyes**	**Tail**	**Pigmentation**	**Hatching**	**Fins**
Sodium arsenate	144hpf									
Control		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$1.5x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$3x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$6x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$9x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$12x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0
$15x10^{-3}M$		0.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0

Table (VI); Statistical analysed studied end point results for sodium arsenate by 48hfp and 144hpf

LOEC and NOEC values table

Substance	End point	NOEC48hpf (Molar)	LOEC48hpf (Molar)	NOEC144hpf (Molar)	LOEC144hpf (Molar)
Retinoic acid	Coagulated eggs	10^{-7}	NO	10^{-8}	$10^{-7.8}$
	Movement	$10^{-7.4}$	$10^{-7.6}$	10^{-8}	$10^{-7.8}$
	Circulation	$10^{-7.8}$	$10^{-7.6}$	$10^{-7.8}$	$10^{-7.6}$
	Extension of tail	$10^{-7.6}$	$10^{-7.4}$	$10^{-7.8}$	$10^{-7.6}$
	Oedema	10^{-8}	$10^{-7.8}$	10^{-8}	$10^{-7.8}$
	Heart rate	10^{-8}	$10^{-7.8}$	NO	NO
	Eye	$10^{-7.4}$	$10^{-7.6}$	$10^{-7.8}$	$10^{-7.6}$
Lithium hydroxide	Coagulated eggs	$16x10^{-2}$	NO	$4x10^{-2}$	$8x10^{-2}$
	Movement	$16x10^{-2}$	NO	$4x10^{-2}$	$8x10^{-2}$
	Circulation	$16x10^{-2}$	NO	$4x10^{-2}$	$8x10^{-2}$
	Extension of tail	$16x10^{-2}$	NO	$8x10^{-2}$	$12x10^{-2}$
	Oedema	$16x10^{-2}$	NO	$3x10^{-2}$	$4x10^{-2}$
	Eyes	$16x10^{-2}$	NO	$8x10^{-2}$	$12x10^{-2}$
Ethanol	Coagulated eggs	2%	2.5%	1%	1.25%
	Movement	1.75%	2%	1.5%	1.75%
	Circulation	1.25%	1.5%	1.25%	1.5%
	Extension of tail	1.75%	2%	1.5%	1.75%
	Oedema	1%	1.25%	1%	1.25%
	Eyes	1.75%	2%	1.25%	1.5%
	Pigmentation	2%	2.5%	1.5%	1.75%
6-Amino nicotinamide	Coagulated eggs	NO	NO	$1x10^{-6}$	$3.5x10^{-6}$
	Movement	$1x10^{-6}$	$3.5x10^{-6}$	$1x10^{-6}$	$3.5x10^{-6}$
	Circulation	$14x10^{-6}$	$22x10^{-6}$	$1x10^{-6}$	$3.5x10^{-6}$
	Extension of tail	$22x10^{-6}$	$29x10^{-6}$	$1x10^{-6}$	$3.5x10^{-6}$
	Oedema	$1x10^{-6}$	$3.5x10^{-6}$	$1x10^{-6}$	$3.5x10^{-6}$
	Eyes	$3.5x10^{-6}$	$7x10^{-6}$	$1x10^{-6}$	$3.5x10^{-6}$
	Hatching	NO	NO	$7x10^{-6}$	$14x10^{-6}$
Ochratoxin A	Coagulated eggs	$25x10^{-7}$	NO	$1x10^{-7}$	$2x10^{-7}$
	Movement	$25x10^{-7}$	NO	$3x10^{-7}$	$6x10^{-7}$
	Circulation	$1x10^{-7}$	$2x10^{-7}$	$1x10^{-7}$	$2x10^{-7}$
	Extension of tail	$25x10^{-7}$	NO	$6x10^{-7}$	$12x10^{-7}$
	Oedema	$6x10^{-7}$	$12x10^{-7}$	$1x10^{-7}$	$2x10^{-7}$
	Eyes	$25x10^{-7}$	NO	$3x10^{-7}$	$6x10^{-7}$
	Pigmentation	$12x10^{-7}$	$25x10^{-7}$	$6x10^{-7}$	NO
	Heart rate	$3x10^{-7}$	$6x10^{-7}$	NO	NO

Table (A) summary of the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) data after 48hpf and 144hpf for selected endpoints.
NO* *Not observed*

Discussion

Identification of environmental agents that cause damage to unborn children is absolutely imperative. Armed with the knowledge of those drugs, chemicals and foodstuffs that human beings are routinely exposed to that are truly teratogenic, exposures can be avoided, reduced or limited, and the incidence of tragic teratogen-induced birth defects drastically lowered. The burden of this goal currently rests heavily upon animal-based testing, but none of the animal model fulfilling the ideal teratology animal model criteria such as producing large litters after a short gestation, inexpensive maintenance, and an inability and unwillingness to 'bite, scratch, kick, howl or squeal' (Wilson, 1975), and also due to some ethically limitations it is not possible to perform extensive teratological testing on routine basis. Thus, a need exists for assays that retain the high value of in vivo toxicity testing, but can be rapidly performed on a large number of chemicals. Since the thalidomide catastrophe scientists are exploring new alternative for teratological testing (McBride 1961; Lenz 1966). In the recent times zebrafish emerge as a competitive laboratory animal model. In the present study zebrafish embryos were used to test the teratogenicity of some potential human teratogens. Chemicals with known potential teratogenicity to human were selected from different categories of chemicals to study the potential of zebrafish model for teratological studies. Comparisons of the results were done with other laboratory animal species as shown in the Table (B).

Species	**Substances**							
	Retinoic acid	**Lithium hydroxide**	**Ochratoxin A**	**Ethanol**	**6-Amino nicotinamide**	**Arsenic oxide**	**+/+-/-**	**+%**
Mouse	+	+-	+	+	+	+	5/1/0	83%
Rat	+	+-	+	+	+	+	5/1/0	83%
Rabbit	+	-		+	+	+	4/0/1	80%
Hamster	+		+		+	+	4/0/0	100%
Primate	+	-		+	+-		2/1/1	50%
Pig		+-		+	+		2/1/0	50%
Zebrafish	+	+	+	+	+	-	5/0/1	83%

Table (B)
"+" positive teratogenic results
"-" negative teratogenic results
"+-"unknown teratogenic results

Retinoic acid is expressed in a gradient along the anterior-posterior axis of the zebrafish. Different Hox genes are expressed along the anterior-posterior axis according to the concentration of RA present in that region, embryos showed truncation at anterior and posterior axis as shown in the results because this exogenous retinoic acid altered the natural gradient, causing ectopic expression of Hox genes. Regions that would normally have a lower concentration of retinoic acid now had a higher concentration and hence expressed genes similar to those

expressed in the hindbrain and trunk region of the embryo, where the largest concentration of retinoic acid is usually found. The magnitude of the effects of treatment with exogenous retinoic acid appeared to be concentration dependent Embryos showed lack of movement, lower heart rate, microphthalmia to anophthalmia, short tail and yolk sac oedema which may be indicative of their stunted growth and a less that normal level of metabolization of the yolk sac nutrients. The developmental abnormalities caused by RA among the zebrafish embryos were very much obvious at early stages (48hpf) and later stages (144hpf) of development on the anterior (craniofacial) and posterior (tail) trunk. The phenotypic developmental abnormalities of anterior and posterior trunk of the zebrafish embryos are similar to those observed in human i.e. defective ears, absent or small jaws, cleft palate, aortic arch abnormalities and abnormalities of the central nervous system, and in mice i.e. axial truncation, reduction in the size of first and second pharyngeal arches which normally form the jaws, ears and other facial bones (Gilbert, 2003; Ligas, 2000; Koren G, 1994). Abnormalities of the CNS in the zebrafish embryos can be correlated to the rapid or reduced movement of the embryos (Behra et al., 2004; Bretaud et al., 2004; Granato et al., 1996; Lam et al., 2005; Mckinley et al., 2005; Tiedeken et al., 2005). In the present discussion comparison of the anterior trunk abnormalities of the zebrafish embryos are made with the phenotypic craniofacial abnormalities of the other lab animal models, coagulation with low birth rate, still birth and high infant mortality and in general anterior and posterior trunk malformations with stunted growth of the new born.

Lithium hydroxide treatment affected the anterior-posterior morphogenesis of zebrafish embryos such that higher concentrations of this substance lead to increasingly greater inhibition of anterior and posterior development. Lithium-induced teratogenesis has been shown to perturb the development of ventral structures in premidblastular zebrafish, when the blastula is still forming morphogenetic gradients of proteins in the Wnt signalling pathway (such as b-catenin) and continuing to transcribe maternal mRNAs. In these early embryos, lithium teratogenesis leads to the expression of bustled and radialized phenotypes, which exhibit hyperdorsalization (Stachel et al., 1993). In the sphere-stage embryos, morphogenetic gradients have already been established, so the effect of lithium in this case probably occurs downstream of these events. The phenotype of LiOH cultured embryos higher then $3x10^{-2}$M exhibited severe yolk sac oedema, microphthalmia, short tail, scoliosis, under developed fins and lack of movement. Embryos cultured in $8x10^{-2}$M and higher concentration of LiOH showed high mortality. Similar developmental malformations induced by lithium are observed in other vertebrate species e.g. in mice i.e. reduction in the number of new born ,vertebral defects, cleft palate, craniofacial abnormalities (Chenroff and Kavloc,1982; Szabo,1970; Smithberg and Dixit,1982; RTECS,1995a), in rat i.e. malformations of eyes, external ears, cleft palate, reduction in the number of live birth(Wright et al., 1970; Marathe and Thomas,1986), in pig i.e. reduction in the live piglets numbers, still birth and high mortality among the live piglets(Kelley et al., 1978), and in human i.e. Ebstein's anomaly ; a congenital cardiac abnormality, still birth and other developmental defects in the Apgar Score (The

Apgar score is a standardised way of checking the health of a newborn baby) "*Appearance, Pulse, Grimace, Activity, Respiration*" (RTECS, 1995b).

Blader and Strähle (1998) have studied that embryonic development of zebrafish is affected by ethanol in a manner similar to higher vertebrates. Phenotypic abnormalities caused by ethanol were observed among the zebrafish embryos cultured higher than 1% ethanol solution as shown in the Table (A) by LOEC and NOEC values. The effected range of ethanol in the present study is significantly different with Blader and Strähle´s (1998) ethanol concentration 2.4 percent. There was high mortality rate among the zebrafish embryos cultured higher then 1.5% and this is may be due to apoptosis by activating the cell's self destruction machineries as suggested by Sulik et al. (1988). Microphthalmia and anophthalmia was observed in embryos treated with 2% and 2.5% ethanol, supporting the fact that ethanol does affect eyes development among zebrafish embryos (Blader and Strähle, 1998). Ethanol did cause defective development in the posterior structures in zebrafish embryos. Although the mechanisms behind the observed deformities of the trunk and the tail are not known, it can be conjectured that the impairment of epiboly may be responsible. Blader and Strähle (1998) noted that epiboly was marginally impaired by the treatment, with the advance of the blastoderm margin lagging behind untreated controls by 10 to 15% toward the end of gastrulation. Epiboly establishes the posterior area of the embryo including the trunk region and the tail bud and, therefore, it seems reasonable that impairing the last 10-15% of epiboly may cause defective trunk and tail (Gilbert, 2003). Lack of pigmentation was observed among some the embryos treated with higher concentration of ethanol. The mechanism for this lack of pigmentation is not known but it is thought that ethanol might cause deformities in the spinal cord from which neural crest cells destined to become melanocytes arise (Gilbert, 2003). These observed developmental abnormalities are similar with abnormalities observed in human, chicken and rat i.e. metal retardation, behavioural abnormalities, craniofacial malformation, heart and neural crest defects (Gilbert, 1997; Cartwright et al., 1995; Smith, 1997; Sulik et al., 1988; Koren G, 1994).

6-AN $3.5x10^{-6}$M rearing solution and higher concentrations caused high mortality, low hatching rate, lack of movement, affected circulation, microphthalmia, short to no developed fins and short tail. It can be conjectured that 6-AN inhibition of nicotinamide adenine dinucleotide dependent reactions during ATP synthesis is responsible for these malformations. When 6-AN decreases the pentose pathway then the arterioles partially lose their ability to regulate the blood flow and blood resistance increases then all the normal mechanisms and metabolisms of the body effected and this may in turn produce malformations in the developing embryos (Wilson et al., 1975; Dietrich et al., 1958). This malformations pattern is similar with that observed in mice i.e. decrease litter size, CNS problems (Matschke et al., 1997), cleft palate, hind limb defects (Pinsky et al., 1960), in rat i.e., stunted growth, foetal death, craniofacial abnormalities, CNS problems (TJADAB, 1975), in rabbit i.e. stunted growth, cardio vascular problems, CNS problems and craniofacial including eyes and ears defects (TJADAB, 1983).

Zebrafish embryos cultured higher then $1x10^{-7}$ rearing media of OA develop malformations. By day six these embryos presented high mortality and affected circulation and spontaneous movement, yolk sac oedema, microphthalmia and under developed fins. These malformations may be due to chromosomal aberrations because it can cause cell death, retardation of differentiation in developing embryos (Wilson, 1973; Manolova et al., 1990). Another possible way of explanation for these malformations is OA-DNA adducts in the zebrafish embryos body tissues (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998). These observed malformation pattern is similar with that observed in rat i.e. high mortality, decrease in the number of new born, decrease foetal weight, celosome with oedema and CNS abnormalities (More´ and Galtier, 1974; Kinhara et al., 1984), and in Mice i.e. high mortality craniofacial malformation, microphthalmia, anophthalmia, malformed tail and limbs (Singh and Hood, 1985; Arora et al.,1981).

No arsenic related mortalities or deformities were observed in any of the treatments performed. There were no notable mortalities or deformations were observed at any experimental concentrations among the zebrafish embryos at 48hpf and 144hpf. These results are opposing to the other lab animal results because arsenic is a proven teratogenic substance as studies have shown that arsenic can cross the mammalian placenta, affecting developing embryos whose mothers undergo exposure (Wlodarczyk et al., 1996). One possible reason for the arsenic's apparent lack of effect on zebrafish development is that neural patterning defects in vertebrates, such as those associated with arsenic exposure, are notoriously difficult to detect from morphological observation alone (Furutani-Seiki et al., 1996). The neural tube forms by the process of apoptotic cavitations in fish, as compared to neural groove invagination in mammals, and as a result, makes gross morphological observation of neural tube defects much harder to recognize. Arsenic exists in trivalent and pentavalent ionic forms, and is often given as either sodium arsenate (As(V)) or arsenic trioxide (As(III)), with sometimes opposing results. Some studies have suggested that As(V) is less toxic to fish than As(III), an identical result to findings in higher vertebrates (Dabrowski 1975). This may explain the absence of abnormalities as in this study As(V) was used for the experiment. But this also opposes the phenomena that only the trivalent ion of arsenic has direct biological ramifications: As(V) must be converted to As(III) to be active (Peterková and Puzanová 1974). Kalter (1968) noted that the effect of teratogens in organisms such as fish and amphibians is primarily dependent upon the stage at which the embryo is exposed to the chemical. Response to arsenic is also species dependent and therefore cannot be generalized based on broader classifications.

Results and the above discussion show that the zebrafish embryo toxicity test has provided positive evidences about the teratogens testing. This test is simple to handle, easy to understand, economical and easily repeatable. In the present study the percentage of positive results is high (83%) as shown in the Table (B) and comparable to other laboratory animal species. But there are a lot of advantages with this animal model, i.e. this specie is easily obtainable, inexpensive, has high

fecundity, a small size (3 to 5cm), short generation time, rapid development, external fertilization, translucent embryos and is easy to maintain at laboratory level (Laale, 1977). Under appropriate conditions one female lays approximately 50 to 200 eggs per day (Laale, 1977), so the sample size can be large enough as desirable. The zebrafish comes close to being the ideal model organism for vertebrate developmental studies because it appears to combine the best features of all the other models. Like most of the amphibians, zebrafish embryos develop externally and can be viewed and manipulated at all stages. However, zebrafish development is more rapid than most of the amphibians and the organization of the embryo are simpler and (like worms and fruit flies) the embryo is transparent. Like the mouse, the zebrafish is amenable to genetic analysis and has a similar generation interval (2-3 months). However, zebrafish are smaller than mice and they produce more offspring in a shorter time. A female zebrafish can lay up to 200 eggs per day, while a mouse may produce a litter of up to 15 embryos in 21 days.

Most of the proof-of-concept studies performed so far have shown that known teratogens (e.g. Valproic acid, cadmium and tetrachlorodibenzo-*para*-dioxin etc) in humans can be recapitulated in the zebrafish (Rubinstein, 2006). It still remains to be proved, however, whether drugs with unknown toxicity characteristics will cause effects in zebrafish embryos. Studies with wider range of compounds with unknown toxicity characteristics will allow toxicologists to predict with reasonable assurance that such effects are likely in humans. Furthermore, it may be difficult to correlate the concentration and absorption of these compounds by zebrafish embryos in the media required to cause toxic effects in zebrafish to the plasma concentration of compound that would cause the same effect in humans. Determination of the actual amount of compound absorbed by zebrafish as well as an understanding of compound metabolism in zebrafish will be important to make useful correlations. Potential differences in the physiology of embryos should also be considered. Based on the results of this preliminary study, it can be said that in future with more extensive research and with wider range of compounds zebrafish assay can be integrated into teratological and drug toxicity screening programmes on a commercial level for the drugs and chemicals before human use. This work has been a necessary first step toward developing zebrafish as a model to predict toxicity of compounds in humans.

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