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Daniorerio, as a Model for Embryogenic Toxicity Study: A Review

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Abstract : According to the animal welfare legislation, the fish embryo model is not regulated by recent established enactment. It is considered as a substituent of animals such as rodents. The Zebrafish (*Danio rerio*) is the most promising oviparous vertebrate model for the identification of wide ranges of human diseases and discovery of drugs. Due to the transparency of embryos, genetic resemblance with mammals, short reproduction cycle as well as the low cost and ease of maintenance, this ex vivo vertebrate model has been widely used for the developmental neurotoxicity study. Fish embryo exemplanary is a significant model for the assessment of chemicals in the environmental toxicity study. This aquatic model is also useful for understanding the proper mechanisms of toxicants in the adverse and long term effects of developmental organogenesis. For numerous advantages and significance of using this oviparous model it provides several opportunities in the field of research in future.
Key words : Zebrafish, neurotoxicity study, environmental toxicity, acute fish test.

Introduction

Zebrafish (*Danio rerio*),an ex vivo model, which is extensively used to assimilate an overall idea about the level of cellular as well as molecular pathogenesis of various diseases in human beings [1].This non-human model system is a worldwide important vertebrate toxic exemplary for the purpose of new drug discovery and several approaches of drug therapy.[2]This facile *Daniorerio* model is significant for the purpose of genetic research because of the presence of the genes which are orthologs between this species and humans. In the year 1970, zebrafish was used as a model for genetic screening which was resembled as the first assay of vertebrate but recentlythis non-human vertebrateminiature is also used for the study of organ development. The embryogenic developmental study of the *Daniorerio* was described by the various organ developmental toxicity studies and which also affected by the ecoenvironmental pollutants including toxicants such as Heavy metals, Organochlorienes, Synthetic aromatic hydrocarbons, Natural estrogens, Endocrine disruptors. [5]The industrial hazards are the toxic chemicals which may incorporate in the air, water and soil and suspected as the neurotoxicants. [3, 4, 5]The neurotoxicants particularly damage the motor neurons of the caudal embryo in the

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third region; as a result the movement of the embryo will be defected. Chemical toxicants are more sensitive in the developing brain of the embryo than the brain of the adult; causes mental retardation in the developed embryo. [3]

For the embryogenic toxicity study, the embryos of this oviparous model is chosen, depends on some characteristics. These are – (a)The transparency of embryos.[1, 2, 3, 4, 6](b) Zebrafish quickly develops at the temperature of 26ºc[4] and within the 3 months the embryos become mature.[3] (c) The development of neurons and the tract of axon may usually visible in the live embryos by using green fluorescent protein(BODIPY TR Methyl Ester Dye) [7]or various markers in the primary embryonic cells in the Danio rerio. [8](d) This oviparous species can be easily obtained and its ease of maintenance in a closely packed recirculating system is more advantageous. [1, 2, 3](e) Under the appropriate environment, the female species lays a large number of eggs approximately 50-200 eggs per day which are non-adherent in nature.[3, 4](f) Small in size (5 cm for adult, 5 mm for larvae of 7 days post-fertilization). [1, 2, 3, 4](g) The developed embryos are exutero type. [5] (h) Zebrafish embryos can be uninterruptedly bred throughout the year. (i) Short breeding life cycle.[3, 4, 5]Cell lines can be visually traced throughout the first week of the development stage. After one week from the histopathological section of larvae the specific cell fates are traced by using confocal microscopy[2]and Differential interference contrast microscopy. [3]The neurons of the specific types can be traced in whole fixed embryos by the process of insitu hibridization.[9]The activity of motor neurons can be noticed by the method of calcium imaging and the Patch Clamp recording which are in vivo technique.[10]Generally, the zebrafish is mainly used at a large number in the cell-based assay than using the throughput. The obtained informations are more accurate in this type of vertebrate model than the rodents and other animals. [3]

Zebrafish Using for Ecotoxicological Study –

Ecoenvironmental Monitoring

Environmental pollution is now a worldwide threatens for human health.During the study of ecotoxicity, water, soil and the quality of waste water can be monitored by the assays of *Daniorerio* in a direct manner. [11]Water pollution affects the survival and the breeding cycle of the aquatic species and also affects the human health badly through bioconcentration. [5, 6]This oviparous aquatic miniature has also been used to evaluate the risk and also help to identify the toxicity of the petroleum products or byproducts in the working condition of the petroleum industries. [12] This exvivo model is a significant system for environmental monitoring because of its sensitivity to different contaminants.As a result the change of several characteristics such as morphology, gene expression and physiology, behavioral changes were observed in ecotoxicological evaluation using biological markers[5] (antiacetylated tubulin,antityrosine hydroxylase). [3]In the year 1984, The International Organization for Standardization (ISO) first organized the publication of the zebrafish toxicity test. [5]According to the recent OECD guidelines, the acute toxicity test is under the guideline number 203 (OECD TG-203; Fish acute toxicity test) under which ecotoxicology study belongs.[3]Bioconcentration is a toxicokinetic property of embryos.[6]Several numbers of suggestive provide a significantconcept about the bioconcentration potential of embryo. Differentiation in the bioaccumulation of the chemicals such as phenanthrene and phenanthridine in the embryo of zebrafish exhibitsinter relationship in between the bioconcentration and lipophilicity.[6, 13]Embryogenic exemplary of the *Danio rerio* ismost authentic for their morphological characteristics and specification of various stage peculiarities in bioconcentration study. The yolk of the embryo represents as an additional type reservoir system for the agglomeration of chemicals. The two outer membranes, which are protein in nature, protect the teleost embryo. These two protein membranes are the chorion and the perivitelline membrane which have a thickness of 10μm. [6]According to the Cheng *et al*. 2007; the chorionic membrane of this ex vivo model contains pores which have a size of 0.17μm². These pores restrict the uptake of several large chemical compounds such as fluorescein dextrans which is larger than 3kDa.[14]The uptake rates of the smaller molecules also may vary.Lindane is a molecule which decreases the uptake in zebrafish embryos. In the studyof Gorge and Naget, 1990; comparing with other compounds, the steady state equilibrium of Lindane attain later in the stage of pre-hatched embryos than in larvae and juvenile fish. Chorionic membrane state an inter-relation in between the acute toxicity and embryo toxicity.[6]

Monitoring of Toxic Heavy Metals

In case of toxic heavy metal monitoring both the embryos and the adult zebrafish models are used. The main function of heavy metals is to inhibit the enzyme activity[15] and also the prevention of expression of gene.

[16] Toxic heavy metals can be detected in an indirect manner bysupervising the activity of enzymes in the aquatic environment and in other hand, by the biomarkers of the gene expression in the embryos of the *Daniorerio*.[5] According to the study of Ling et.al, 2011; due to the presence of heavy metals such as cadmium, zinc the activity of the enzymes i.e. Supper Oxide Dismutase (SOD), catalase are inhibited.[17]In the study ofRichelti et.al, 2011; state that the activity of enzyme cholinesterase due to the presence of the heavy metal derivatives such as cadmium acetate and lead acetate was prevented.[5]Mercury chlorideinhibits the activity of adenosine deaminase activity. [18]During the exposure of the heavy metals such as Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} the inducing effects of gene expression of metallothionein in zebrafish can be observed.As a result, the mt mRNA level in zebrafishis decided as a marker to observe Hg^{2+} andCd^{2+}in water.[19]Dvrl expression which is responsible for the asymmetry of left-right gene function in embryogenesis of *Danio rerio* is directly related to the heavy metal arsenic. So, in the zebrafish dvrl expression can be used as a biomarker or end point to evaluate the toxic effects of arsenic. [20]

Monitoring Endocrine Disruptors

The most common endocrine disruptors are polybrominateddiphenyl ethers (PBDEs), dioxin, bisphenol A (BPA) and their derivatives. Binding to the estrogen or androgen receptors they interfere with the function of natural hormones. Endocrine disruptors are responsible for reproductive problems, birth effects, developmental abnormanilities, metabolic disorders and cancer by affecting the synthesis, release, transport and metabolism of endocrine disruptor substances. [5]Polybrominateddiphenyl ethers are a group of environmental pollutant that distorts behavioral effects and also highly responsible for developmental toxicity in this ex vivo model. [21] According to the study of Chen et al, 2012; a commercial mixture of PBDE named as DE-71, affects the retinoid level in the *Danio rerio* by destroying the transport, storage and metabolism. So, retinoid level is the significant biomarker of the endocrine disruptor DE-71. [22] Dioxin inhibits follicle mutation by disturbing estrogen biosynthesis and estrogen regulated signal transduction. Dixon pollution posses a threaten effect in human health. [5] Specific expression of brain due to the enzyme aromatase is the significant endpoint of the compound bisphenol A in the stage of early embryogenesis.[24]According to the study of Gilbert et al. 2011;state that in the otic vesicle of the embryo, BPA induces severe malformations.[23]In the study of Saili et al. 2012;state thatthe at low-dose level of BPA exposure causeslarval hyperactivity or learning deficiency in adult zebrafish. BPA, endosulfan, methoxychlor indicate exposure to mRNA expression in male zebrafish. Therefore, Bisphenol A is important endocrine disruptors with a correlation in between embryo toxicity and teratogenecity. [5]

Monitoring Organic Pollutants

Polyaromatic hydrocarbon (PAHs) pollutant is an important organic pollutant. PAHs derivatives such as dichlorophenol, naphthalene, aromatic pesticides can interfere with the endocrine systems which affect embryonic development, DNA damage and oxidative stress in zebrafish.[25] PAHsposses the developmental toxicity in the zebrafish embryo but also it has the cardiac developmental effects and the expression of genes in the embryos of this ex vivo model. In the study of Zheng et al. 2011; Perfluorochemical derivatives such as perfluorooctanesulfonate (PFOA) and perfluorooctanoic acid(PFOA) have an effect to the development of embryo in *Danio rerio*. This study also gives a conception about thePFOA exposure which may change the fatty acid binding protein gene expression and posses a bad effect of the triglyceride levels of liver. Interference of the perfluorooctanesulfonate with the steroidogenesis which changes the gene expression related to the endocrine in the zebrafish embryos. [5]

Developmental Neurotoxicity (DNT) Study Using the *Danio rerio* Model

Daniorerio, the extensively used vertebrate exemplanary, has been applied for a long period of time in the developmental biology as well as for the investigation of the drugs for neurotoxicity study. The biological similarities between the zebrafish and other vertebrates in the development function of CNS, in the prenatal and postnatal stages, make this oviparous model more advantageous as the research tool for the developmental neurotoxicity assay.[26] In this system blood brain barrier (BBB) plays an important role of protector against the neurotoxic agents. Prevention of the transport of the free substances is the fundamental function of the BBB by the function of filtering using the endothelial membrane. [27] For the screening purpose of the environmental contaminants, this exvivoaquatic model is more prior than the evaluation done by mammals in case of determining the potentiality of the neurotoxic substances.The neurotransmitters and the neuro active substances

are separated by the BBB from others in the CNS and those present in the peripheral nervous system by obstructing the interaction in between the two systems. [3, 26]Neurobehavioral alterations are a key observational end point of the neurotoxicological DNT studies by using the zebrafish model. In the field of research, the changes of neurobehavior in this vertebrate model embryo, can be clearly categorize into three categories such as- spontaneous movement, touch responsive movement and locomotor activity which is induced with the touch stimulation. [38]According to the study of Umans et al. 2012; by the process of immunohistochemical visualization at 48 hpf the presence of the BBB in the early life stage of Danio rerio was evidentially identified. [28] When there is a no clear concept about the maturation of the BBB two tight junction proteins such as Claudin-5 and ZO-1 may exhibit in the endothelial cells at 72 hpf. [29]Some chemicals are responsible for the neurodevelopment deterioration such as Autism spectrum disorder (ASD), Intelligence quotient (IQ) and Attention deficit hyperactivity disorder (ADHD). Many chemical exposures may stimulate changes in the genetic or epigenetic site which modulates the biological systems of various organisms and trigger the alteration in the neurodevelopmental study.Various chemicals including few specific metals such as arsenic, methylmercury and lead resemble as the developmental neurotoxicants. These toxic substances are non-biometals in nature which have several effects in the neurodevelopmental alteration in the developing organisms. The developmental stages of embryos of the *Danio rerio* are well studied and they help to provide guidance to the researchers to find out the major alterations during the assay of the developmental toxicity.[26]

Arsenic

It is natural metalloid element which exists in various organic and inorganic formswith various oxidative states such as trivalent (arsenite) and pentavalent (arsenate) states. [11]In the study of Li et al. 2009; during the exposure period of 4- 30 hpf, at the level of concentration 2mM or above arsenic gives a reducing effect in the reflexive moment frequency under the light stimulation as a endpoint of behavioral alteration in the zebrafish model.[32]The axonal tracts of the embryos of the *Danio rerio* were visualized in the immunostaining experiment, by using the antibody named α-tubulin (α-AT) and reported the interrupted growth of the axon in the brain and the spinal cord region of this vertebrate by comparing the control treatment with the 2mM of arsenite solution.[26] According to the Wasserman et al. 2014; in the United States (US), the IQ levels of the 272 school-aged children are disrupted due to the exposure of arsenic through drinking water at home. During the comparison between the low level of arsenic exposure (concentration of arsenic in water is below 5μg/L) with the high level of arsenic exposure (concentration of arsenic in water is below 5-10 μg/L) the researchers may compare the IQ scoring of the large number of children. Arsenic in the high concentration level remarkably decreases the ability of the overall intellectuality level.[30] According to the study of the Nahar et al. 2014; the researchers found that there is a relation in between concentration of arsenic in urine and as well as the remarkable decrease in the non-verbal IQ, but not in case of the verbal IQ. [31]

Methylmercury

Mercury (Hg) is a very common metal in the environment. We can found Hg in various oxidation states such as elemental (Hg^0), mercurous (Hg^{1+}), and mercuric mercury (Hg^{2+}). The mercurous and mercuric states can interact with the compounds containing carbons as a result methylmercury and ethyl mercury may form. According to the study of the Peterson et al. at the concentration of 100ppb (~ 0.5 μM), the methylmercury, interrupted the neurological development by altering the global expression of genes and their functioning. In the study of the Zhang et al. methylmercury affects the axonal growth or the growth of the nerve by decreasing the density of the tracts of the axon within the exposure period of approximately 2-36 hpf. [26]According tothe study of the Ho et al. 2013;methylmercury(MeHg) is another neurotoxic substance, which affects the normal cell death (apoptosis), responses of the oxidative stress, transcriptional elongation in zebrafish model by altering the expression of genes related to the brain at the level of concentration 60μg/L and the exposure time of 48-72 hpf.[35]In the study of the Hassan et al. 2012; the researchers monitored a remarkable decrease in the proliferation of cells occurring in the neural tube of this vertebrate model which was exposed to 10,50,80 μg/L MeHg at the exposure time of 30 hpf by using PCNA staining. [33] At the protein level the exposure of methylmercury (MeHg) on the developmental stage of Danio rerio was identified by using iTRAQ (isobaric tags for relative and absolute quantification). [34]

Lead

Lead (Pb) is a metallic substance exists in the oxidation states such as +2 and +4 as organic and inorganic form. Due to the exposure of the lead various behavioral alterations are reported by the researchers. In the developmental toxicity study, due to the exposure of Pb, the transmission of signals from the excitatory region and the functions of learning and as well as the memory functions are also altered. Exposure of lead also altered the functions of glutamate receptors or its subtypes [e.g. - N-methyl-D-aspartate (NMDA)].[26] According to the study of Rice et al. 2011; at the concentration of 10 nM or above, the *Dani orerio* model, showed the interrupted startling behavior under the influence of the stimulus of tapping within the exposure period of <2–24 hpf. Therefore, the lead exposure causes the neurobehavioral alteration in this developmental non vertebrate model. [36]In the study of Chen et al. 2012; at the concentration of 0.1 mg/L or above,0.025 mg/L or above, this exvivo vertebrate model, affects the spontaneous movement, interrupt the swimming activity in the stimulation of light or light and dark condition respectively.[37]Recentlythe researchers found the effects on the GABAergic system of embryogenic *Daniorerio* during the exposure of lead acetate at the level of concentration of 100ppb by using the methods ofqPCR and High Performance Liquid Chromatography (HPLC).[26]There are five chemicals (example- Atrazine, 2, 4-D, DDT, Dieldrin), one teratogen (TCDD), one negative neurotoxic compound (melathion) which treat the zebrafish model for the visualization of brain region at the axonal tract.[3]

Table-1: Effects of seven neurotoxicants on the developmental neurotoxicity study [3]

Compound	**Effects on Zebra Fish**
Atrazine	✓ Increase death of brain cell
	✓ Causes disorganized growth of axon in motor region
	✓ Several circulatory problem ; heart and trunk edema, decrease of heart rate
	✓ Bent caudal embryo
Dieldrin	✓ Increase death of cells in brain
	✓ Causes motor neurons in disorganized form in the caudal embryo
	✓ Possess teratogenecity at 48hpf but not at 96hpf
2,4-D	✓ At high concentration increases cell death in brain
	✓ At high concentration disrupt the growth of motor neuron
	✓ Decrease the motility of embryo
	✓ Slightly produces teratogenecity
	✓ Under-developed jaw
DDT	✓ Increase the cell death in brain
	✓ Increase random motion in the embryo
	✓ Hemorrhage in rare cases at the concentration of 1mM
	✓ Possess teratogenecity at 96hpf
Melathion	✓ Possesteratogenecity at 48hpf but not at 96hpf
	✓ Produces short body of the embryo
	✓ Decrease the growth of motor neurons in the caudal embryo
TCDD	✓ Produces high teratogenecity
	✓ Decrease motility
	Brain necrosis
	Heart edema at the concentration of 0.1mM
	Short body

In the developmental neurogenic study we can develop several bioassay methods for the screening and testing of the environmental neurotoxic substances. The end points of these assays are mainly based on the potential effects of the neurologic study. Generally seven environmental neurotoxic substances (2, 4-D, Atrazine, DDT, Dieldrin, Malathion, Nonylphenol, TCDD)are assessed by using the bioassays for testing the embryogenic development.[3]

Evolutionary Parameters of DNT Study in Zebrafish Model-

Teratogenecity

An evaluation of developmental toxicity study can be visualized by scoring various parameters at the period of either 48 hpf or 96 hpf. Teratogenic Index (TI) is the developmental endpoint in this neuronal assay. For the mortality and the developmental malformation the therapeutic index is calculated by the generation of dose response curves. The dose at the 50% mortality (LC_{50}) can be compared with the value of the dose of 50% developmental malformation (EC_{50}). The value of the therapeutic index can be represented as the ratio of LC_{50}/EC_{50}.By using the value of the therapeutic index neurotoxicants can be placed comparing with their relative teratogenecity.[3] According to the Zhang et al. 2003;the measured teratogenecity in the *Danio rerio* posses an interrelationship with the scoring functions of the mammals in the developmental toxicity study.[39] The determination of the LC_{50} values for each compounds were done by exposing this oviparous vertebrate model in the wide range of compounds at every 24 hr until the exposure period of 96 hpf. The estimation of the LC_{50}value was calculated at 48 and 96 hpf for each compound where 30 zebrafish were scored per concentration. At first, the researchers tested five concentrations such as 0.01, 0.1,1,10,100 μM. For the necessity of generating lethality concentration curves higher and lower concentration of compounds were tested. DMSO at the specific concentration where this exvivo model can be exposed was treating as the control. Tests were calculated using Cochran Armitage χ^2 test in the percent lethality concentration curves. The LC_{50} value then calculated by using probit model.[3, 4]

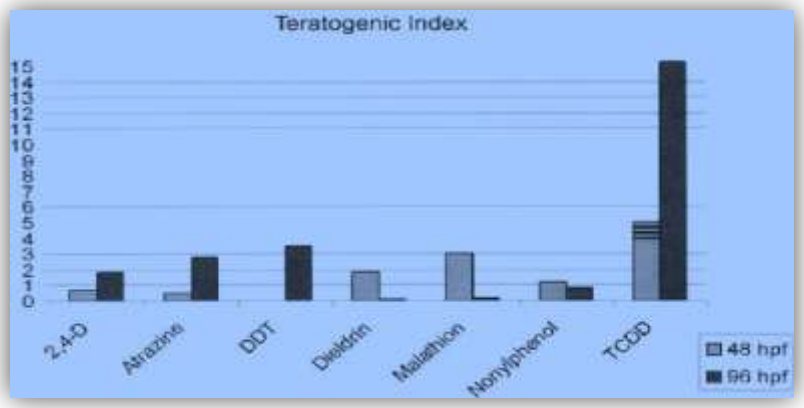

Fig-1: Comparative teratogenecity study of seven compounds at 48 and 96 hpf[3]

In the study of Ton et al. 2006; the researchers found that at 48 hpf, the neurotoxicants such as Dieldrin, Malathion and TCDD were the most teratogenic substances. The estimated value of the therapeutic index of TCDD was greater than 1. At the 96 hpf, Atrazine showed slight teratogenic effects whereas DTT and TCDD were highly teratogenic in nature. [3]

Developmental Scoring of Malformation, Generation of $EC_{50}$and Estimation of Teratogenecity

According to the study of Zang et al. 2003;five zebrafish were scored for the exposure of compounds and the control in the study of the developmental end points at each concentration. The developmental malformation index at every concentration can be calculated by the total number of positive scores which is divided by the total number of parameters. There are several developmental endpoints which were scored at the 48 hpfand 96 hpf.[39]

- **Heart rate-** As the heart rate is highly sensitive to the environmental temperature; the heart rate was firstly counted by using MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2mM Na_2HPO_4) and methylcellulose. For 15 seconds the beat rate of ventricle of each zebrafish was measured for each animal using a counter and a stopwatch and for obtaining the beats calculated per minutes the value is multiplied with 4. [3]
- **Circulation-** The movement of blood cells through the heart and vessels are visually examined. The rate of flow of blood and the pattern of blood flow were also examined visually. Abnormal circulation such as

slow and fast circulation find out the defect of circulatory pattern i.e. circulation only in the head or trunk. [3]

- **Edema-** In the surrounding of intestinal space of a tissue or organ, edema is visualized as fluid accumulation. Circulatory and excretory problems occurred in case of heart and trunk edema. [3]
- **Number of Red Blood Cells (48hpf only) -** The RBCs of this oviparous model have been seen circulating in the transparent blood vessels. The abnormality was scored when the number of RBCs decreases in the circulation. [3]
- **Brain Necrosis-** The nature of normal brain tissue is transparent. As a result of necrosis a cloudy, white or brown areas are visualized in the brain. One or more than one necrotic areas are posses as the positivity in developmental malformation scoring. [3]
- **Hemorrhage-**Due to the malformation of blood vessels or rupture of tissues after the exposure of compounds the hemorrhage is occurred. In zebrafish, a poll of cluster of blood in an area at the site of normal circulation, hemorrhage is visualized. [3]
- **Caudal Embryo Morphology**-Bent or misshapen caudal region of the embryo is the abnormality. This type of defects produces problems in the muscle development and the nervous system. [3]
- **Motility**- Motility is measured to evaluate the disorders of the neuronal and muscular region. Because of touching of the caudal region of the embryo a dissecting needle was used. As a result of touch a rapid swim response is observed which indicates the defects the transmission in the neuromuscular region. [3]

The value of EC50 determinationis symmetrical to the statistical analysis of the calculation of LC50 at each concentration after obtaining the value of developmental malformation index (DMI). [3]

Conclusion-

Daniorerio, a fresh water experimental exemplanary, plays a very significant role in the field of developmental biology, genetic expression of organogenesis and as well as the identification of various diseases and biomedical research purpose. [1, 2] Due to the small size of the embryos of this ex vivo modelthe culturing technique during the first week of their life stage is ideal for the discovery of various diseases. This experimental process states that zebrafish is a very authentic model for the initial assessment of the effects of compounds and their developmental neurotoxicity. This vertebrate model is also a good model for the assessment of teratogenecity study to identify specific effects of neurotoxicity. [3] The embryogenic toxicity study for the *Danio rerio* represents an attractive powerful model for the assessment of the environmental toxicity study. It provides steps for the further research of the identification of the biological indicators as well as molecular markers are to identify the mode of action and the effects of the long term toxicity to the embryos. Zebrafish model is a disease offering experimental model which strengthen the experiment of developmental neurotoxicity study (DNT)[6]along with other human disease to improve the overall idea about the pathogenesis of the diseases and develop several new therapies for the treatment of such diseases. To make the process of DNT more improving, studies of exposure kinetics of several compounds are used in thisnon-human vertebrate embryo model. The effect of chorionation and dechorionation at the time of exposures of chemicals also affect the absorption of chemicals by the embryos. The future prospect of this ex vivo model is to apply them for the screening purpose of developmental neurotoxicants and also for the understanding the mechanisms of the developmental neurotoxicants (DNT).

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