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Review

The posterity of Zebrafish in paradigm of in vivo molecular toxicological profiling

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ABSTRACT

The aggrandised advancement in utility of advanced day-to-day materials and nanomaterials has raised serious concern on their biocompatibility with human and other biotic members. In last few decades, understanding of toxicity of these materials has been given the centre stage of research using many in vitro and in vivo models. Zebrafish (*Danio rerio*), a freshwater fish and a member of the minnow family has garnered much attention due to its distinct features, which make it an important and frequently used animal model in various fields of embryology and toxicological studies. Given that fertilization and development of zebrafish eggs take place externally, they serve as an excellent model organism for studying early developmental stages. Moreover, zebrafish possess a comparable genetic composition to humans and share almost 70% of their genes with mammals. This particular model organism has become increasingly popular, especially for developmental research. Moreover, it serves as a link between in vitro studies and in vivo analysis in mammals. It is an appealing choice for vertebrate research, when employing high-throughput methods, due to their small size, swift development, and relatively affordable laboratory setup. This small vertebrate has enhanced comprehension of pathobiology and drug toxicity. This review emphasizes on the recent developments in toxicity screening and assays, and the new insights gained about the toxicity of drugs through these assays. Specifically, the cardio, neural, and, hepatic toxicology studies inferred by applications of nanoparticles have been highlighted.

1. Introduction

Toxicological studies hold significant importance in the realm of biomedical research. It plays an important role in the evaluation of drug efficacy in preclinical investigations. A part from that, Along with the epidemiological research, the toxicity analysis also ensures the safety of new biochemical compounds to be deployed as drugs, pesticides, food additives, and general use in industries [1]. The safety analysis and the

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toxicological characterization of the biochemical product provides additional values to the determination of industrial outputs. In late 20th century, the catastrophe of thalidomide generated serious attention to toxicity assessment because of the observance of serious birth defects in thousands of children worldwide. Due to this incident, many countries of the world started to look into the science of toxicity testing and teratogenicity, caused by the toxins produced in nature [2]. Accordingly, the production of around 40% to 80% of the biochemical compounds used in the field of pharmaceutical drug discovery were halted. However, in an investigation of the pharmaceutical industry, a comprehensive preclinical- to-clinical database indicated a good specificity (>80%) which showcased that the false positive predictions of toxicity are low in the studies of preclinical toxicology but at the same point due to the low sensitivity (<50%), unpredicted human safety events were observed [3]. If the toxicity assessment of various natural and unnatural biochemical compounds is not performed via comprehensive preclinical- to-clinical trials, then those substances may start to affect human health negatively. Along with the prevention of the disease, the toxicity assays also focus on the reduction of the misuse of agents like alcohol, drugs, and chemical byproducts for industrial expansions [4]. Hence, the improvement and advancement in toxicological investigations and research can lead to the mitigation of losses by revealing the toxic mechanism of biochemical compounds. Considering these facts, this section focuses on the numerous techniques and assays which explain the importance of toxicity analysis and its impact on significant model systems (in vivo and in vitro).

1.1. Animal model system in toxicity assessment

In contemporary biological studies, the contribution of model organisms has been significant [5]. The common animal model organisms used in toxicity assessments are: Free-living soil roundworm (nematode) (*Caenorhabditiselegans*), Fruit fly (*Drosophila melanogaster*), Frog (*Xenopuslaevis*), Zebrafish (*Danio rerio*), Chick, Rat, Laboratory mouse; Sea urchin, Sea slug (*Aplysia*) and the puffer fish. Among the above-mentioned species, *C. elegans*, Drosophila, Zebrafish, and mice are selected primarily because of their likeness to humans in terms of physiology, genetics, and anatomy coupled with the limitless sources and simplicity of manipulation. Along with the toxicity assessment, these animal models are also used for the scientific investigation related to metabolism, distribution, absorption, and elimination, commonly known as ADMET analysis [6].

1.2. Fish as a model organism for screening toxicity

Affiliated to the Kingdom Animalia and classified into *Phylum Chordata* and *Subphylum Vertebrata*, "fish" plays a precise role in demonstration of human's physiology. Toxicity screening of pollutants on fish became relevant due to the alterations in their biochemistry, physiology, morphology, and/or genetics. The toxicological effects may influence the precise stimuli development, growth, and reproduction [7,8].

1.3. Significant features and chronological perspectives

Zebrafish (*Danio rerio*), a small benthopelagic cyprinid (teleost) fish affiliated to the family *Cyprinidae* in the class *Actinopterygii* (ray-finned fishes), originated from the riverine habitats of the Himalayan region of South Asia, specifically Bhutan, Pakistan, India, Bangladesh, Nepal, and Myanmar; has arisen as a pivotal model system in various fields, including neurobiology, toxicology, molecular biology, and developmental biology [9] (Fig. 1). The salient features of zebrafish make them useful in assorted biological studies. The rapid in vitro fertilization property of the embryos (or eggs), make them easily accessible for experimentation. The post-fertilization (hpf - hours post fertilization) stage of the zebrafish developmental cycle shows that at 18hpf transparent embryos with well-developed eyes, ears, segmented muscles, and brain are observed. The blastula stage lasts for 3hpf and the gastrulation phase takes place at 5hpf. Segmentation gets completed and the primary organ system is formed within 24hpf. After the hatching period gets completed by 72hpf, the post-fertilization embryo gets out from the eggshell. The rapid transformation of the embryos into the larval stage (or a smaller version of the adult stage) occurs in 4 days. Such rapid development of a model organism substantiates the rapid variations with progressing technology and worldwide manufacturing. The zebrafish model organism can propose real-time in vivo research and studies on prospective threats to human health and nature caused by naturally occurring substances and the commercialization of new products. As a result of the study of those compounds on this model organism i.e., zebrafish, our restricted understanding of the precise effects of environmental exposures was developed [10]. Sharing almost ~70% likeness concerning the human genomes this model organism shares similar crucial body systems such as the neural, digestive, and circulatory systems like human beings [11]. These similarities essentially reinforce the equivalence response to therapeutic agents among the two species [12]. Zebrafish is a model organism that is preferred due to its ability to mimic human diseases both genetically and morphologically [13]. Due to the higher spawning of eggs and a higher rate of fertilization, several strains established for molecular biological determinations might be incompatible with toxicity investigations [9]. Therefore, at the same time precautions should be considered while selecting the wild-type strains (Fig. 2).

2. Toxicity profiling (*In vitro, In vivo*, and Omics - assays)

A toxicological profile is a way to identify the toxic substances present in an environmental specimen. This is done by testing the sample's extract in a series of bioassays. It's like a unique fingerprint for the toxicity of that particular sample. This technique allows the identification and effect of toxicants on various environmental or biological samples in a dose-dependent manner. A toxicity test, by extension, generates data concerning the adverse effects of a toxicant and assesses the portion of risk involving the dosage response. Toxicity screening is very important to assess the therapeutic potential of molecules and also, for the advancement of novel pharmaceuticals [19]. The principle of toxicity profiling is based on checking the effect of a particular sample on laboratory animals, observing its effects on exposure to higher doses, and finally its toxic effect on human health [2,20]. The essence of toxicity profiling is not only about testing the toxicity of a substance but also characterizing the possible toxic effects produced by the toxicant [21]. The critical effect is tapped to develop reference values representing doses below a level that has significant adverse effects. This section highlights the historical importance of toxicity profiling and the contribution of nanoparticles in this field.

Toxicity profiling, according to different considered parameters, entails usually these types [2]:

i. Acute toxicity studies
ii. Sub-acute toxicity studies
iii. Chronic toxicity

Acute toxicity testing is a short-term assessment performed to evaluate potential hazards caused by a single dose of the toxicant which is target organ-specific[22]. They provide safety measures and guidelines for workers involved in the testing procedure by setting up a standard level for toxicants. The sub-acute level of toxicity studies is accomplished to ascertain the impact of various dose levels on specific organs. This kind of study evaluates the characteristics of toxic doses in a more realistic context than acute toxicity evaluations. Chronic toxicity analysis is usually extended over longer periods and they involve relatively larger groups of model organisms to be used specifically determining the organs affected and the carcinogenic potential of a drug.

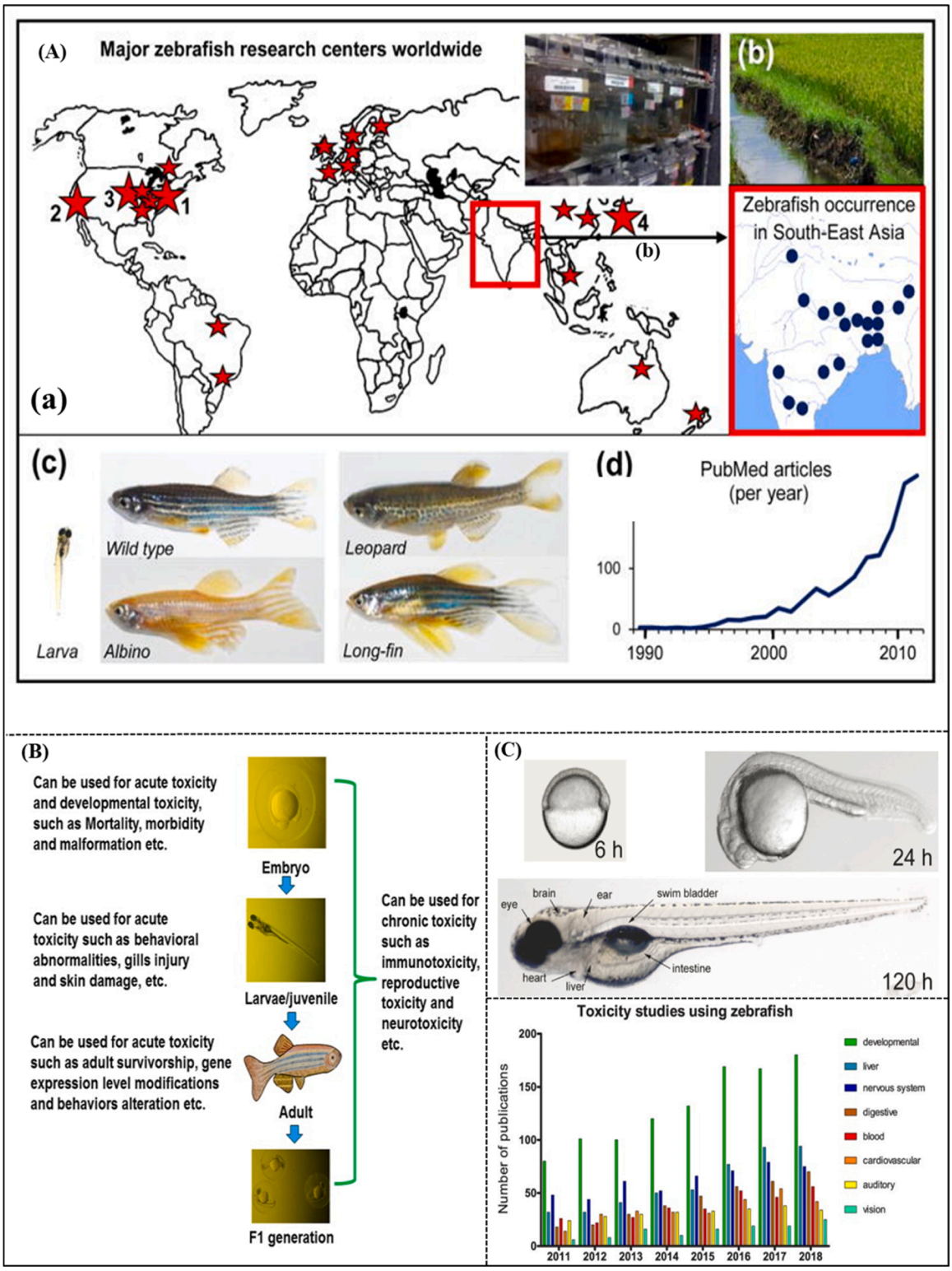

Fig. 1. (A) Zebrafish in laboratory research and natural environments. Panel (a) shows major zebrafish research centers established worldwide (red stars), including the National Institutes of Health, University of Oregon, and Washington University in the USA, and RIKEN Institute in Japan. Inset – a typical rack housing hundreds of zebrafish in a research facility. (b) Typical habitat of zebrafish in the wild (shallow waters, e.g., rice fields) in various regions of Southeast Asia. (c) Larval and adult zebrafish (including several common color variants). (d) The growing number of published zebrafish models (assessed in PubMed in September 2013, using terms 'zebrafish' and 'behavior') [14]. (B) Schematic diagram of nanotoxicological studies relevant to the different stages of zebrafish development [15]. (C) Zebrafish developmental stages. Zebrafish at 6, 24 and 120 h post fertilization (hpf) are shown. By 120 hpf, zebrafish develop discrete organs and tissues, including brain, heart, liver, intestine, eye, ear and swim bladder, also the other figure indicates that Numerous systems can be interrogated for toxic endpoints using zebrafish and the number of publications is trending upward, as indicated by these examples [16,17].

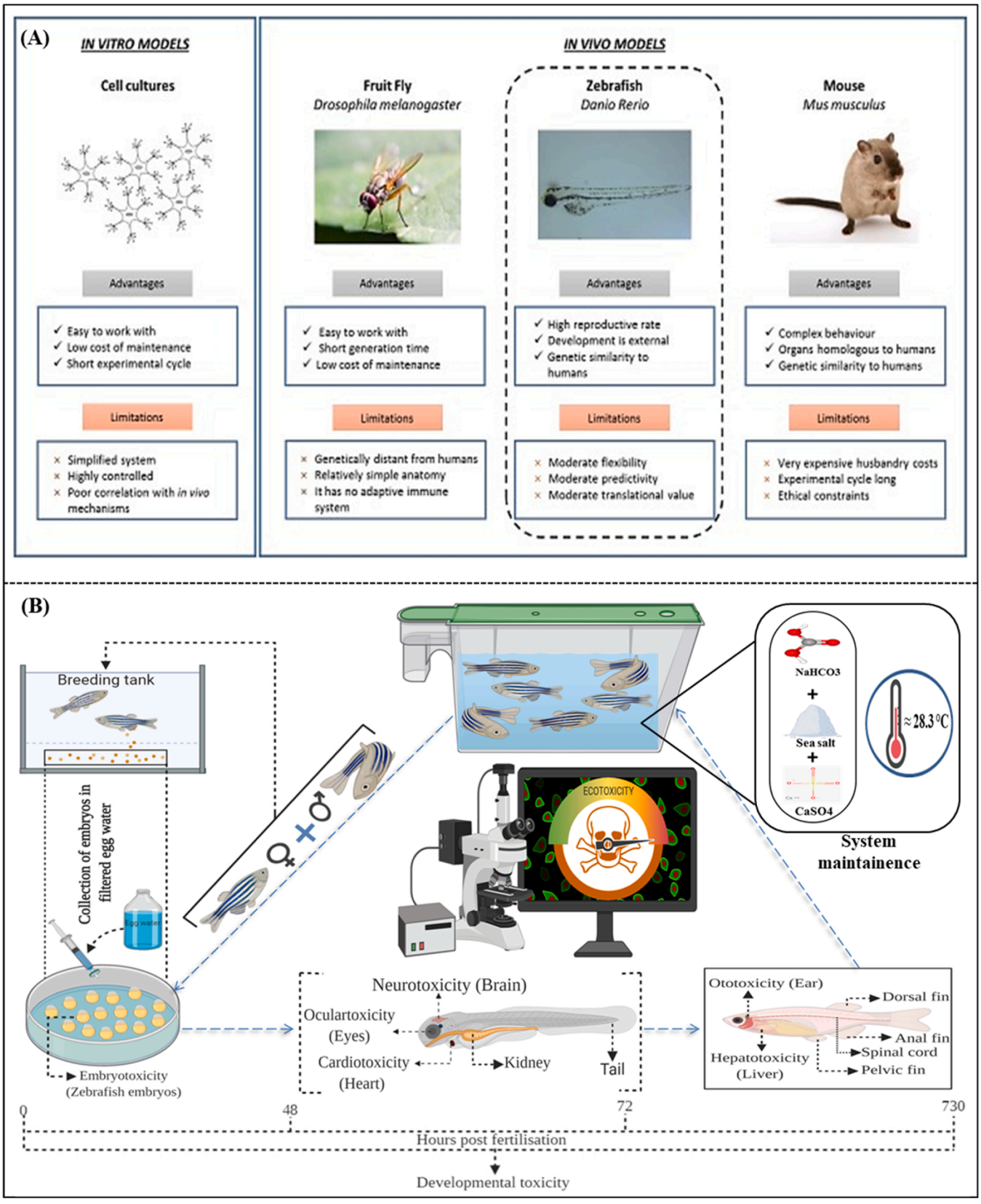

Fig. 2. (A) *In vitro* and in vivo models for developmental neurotoxicity screening [18]. (B) Zebrafish model for various toxicity screening.

2.1. In vivo, In vitro, and Omics strategies for toxicity profiling

In '*In vivo*' toxicity testing, the organisms are exposed to chemicals of interest and adverse effects are observed. The duration of exposure depends on the assay being monitored and depending on the type of toxicity assay. The advantage of using the in vivo technique is that it includes the measurement of absorption, distribution, and metabolism, as all these factors affect the toxicity of the sample. However, there are certain disadvantages as well. At certain points, factors such as sensitivity, interspecies extrapolation, and perplexing factors act as drawbacks. Despite these limitations, in-vivo assays are most frequently utilized for the assessments of hazards to human health [23].

The '*In vitro*' toxicity testing involves bio-analytical assays where whole cells or molecules are exposed to the chemicals of interest and thereafter the adverse health effects (if any) are observed [24]. In-vitro assays are typically carried out on specific cell types depending on the endpoint of interest. These assessments are also restricted to an extent as exemplified by no incorporation of toxico-kinetics. Though they are precise and highly sensitive, they have a lower level of relevance which is another hitch for this assay system [23].

With the advancements in technologies in the fields of molecular biology and bioinformatics, comprehensive study of toxicants allowed the researchers to evaluate the toxic effects of pollutants on different molecular pathways. The immense range of omics technologies surround topics like genomics and proteomics which facilitate the detailed studies of cellular processes of an individual in a community or biodiversity in response to different environmental stimuli [25]. Moreover, techniques like transcriptomics, metabolomics, etc. are being used and further evaluated in investigational toxicology [26]. Research is still ongoing for enabling the utilization of that data in regulatory toxicology. The advances of omics have not only been a boon to the generation of new knowledge in ecotoxicological studies but also paved an innovative dimension for the mechanism-based and system biology-based ecotoxicological approaches [25].

3. General toxicological investigations linking human and zebrafish model

3.1. Developmental toxicity

According to the FDA guidelines, three distinct protocols have been stipulated which focus on reproductive capacity and reproduction (segment I), developmental toxicology and teratology (segment II), and perinatal and postnatal development (segment III) [27]. The Health and Environmental Sciences Institute (HESI) Developmental and Reproductive Toxicology Technical Committee organized a meeting in the year 2008 [28] wherein, the utilization of zebrafish and alternative in vitro models for the study of developmental toxicity was addressed. These groups utilized three distinct efforts via zebrafish embryos, attaining the following results:

a. A joint study conducted by Bristol-Myers Squibb and Phylonix Pharmaceuticals, Inc examined 12 compounds, primarily retinoids, in a blinded manner. The study evaluated the LD50 values in comparison to the lowest-observed-adverse-effect level (LOAFL) for identifying morphological defects in the head, body, liver, or intestine[29].

b. DanioLabs Ltd developed the second zebrafish developmental toxicity assessment and the evaluation was accomplished in a preliminary study by Pfizer, Inc., and ECVAM. The embryos were treated at the 2-cell stage and the assessment of embryos was performed at 24 and 48 hpf. This experiment investigated a total of 18 compounds, which encompassed three categories of in vivo teratogenic potency. The experiment yielded an overall concordance rate of 72% for the correct classification of non-teratogens, weak teratogens, and strong teratogens, additionally, the respective success rates of (67, 100, and 50) % were obtained from the experiment.

c. The Bristol-Myers Squibb Reproductive Toxicology group conducted the third zebrafish assay, which focused on assessing general toxicity. This involved measuring IC50 values in NIH3T3 fibroblasts and LC25 values in zebrafish embryos. A conclusive assay was performed in this approach, wherein the embryonic zebrafish were treated at 4 – 6 hpf and the assessment of the morphological defects was performed at 5 dpf zebrafish embryos. The assessment involved the study of 24 compounds, a combination of ECVAM validation compounds and Bristol-Myers Squibb pharmaceutical compounds that are known to exhibit teratogenic activity. For in vivo teratogenicity data, the overall concordance was 92% (specificity = 86%; sensitivity = 94%).

According to the present global guidelines, developmental toxicity entails administering compounds to pregnant animals, often rats and rabbits, then conducting toxicity evaluations on the developing fetuses [16]. In the early 1980 s, novel approaches for evaluating developmental toxicity emerged. The techniques encompass various procedures, such as conducting in vitro cell differentiation utilizing either immortalized cell lines or primary cells, employing the in vivo frog embryo teratogenesis assay (FETAX), and employing the in vitro whole embryo cell culture test [30]. By following the guidelines and previous experiments related to developmental toxicity assays, the imminent studies, and research may pave a path for the advancement of developmental toxicity studies.

3.2. Reproductive toxicity

The profound insights obtained from the zebrafish genetic and developmental investigation has helped it to be evolved as one of the most prominent vertebrate models for toxicological studies. Traditionally, mice were the most used mammalian model for the reproductive toxicity assays of several drugs and chemical compounds. Histopathological examination of the testis or ovary, evaluation of sperm quality, quantification of egg production, and other related parameters formed the foundation for studying the toxic potential of organisms [31–34]. Regrettably, evaluating reproductive toxicity employing animal models proved challenging, lengthy, and costly. Moreover, when assessing the reproductive toxicity of environmental samples or chemicals, mammalian testing is impractical due to the need for higher doses. As pollutants are typically present at lower concentrations in aqueous systems, only water-soluble chemicals can dissolve in them [35].

Although zebrafish are small in size, their genetic sequences and reproductive functions closely resemble those of humans. This makes them a valuable vertebrate model for studying infertility. Significant features like optical transparency during the embryonic phase and rapid maturation, along with its biological functions made this model suitable for reproductive toxicity studies. Risks related to human reproductive health can also be assessed by demonstrating underlying mechanisms associated with infertility, common between mammals and zebrafish [36]. Zebrafish is becoming a popular choice for studying infertility and reproductive toxicity due to its simple embryogenesis and in-vitro fertilization [37–41] (Fig. 3).

3.2.1. Behavioral Reproductive performance of Zebrafish

The genetic basis of development and salient characteristics of embryogenesis has been widely investigated in zebrafish [43,44] [45]. Male and female zebrafish exhibit distinct mating behavior. Males typically exhibit characteristics such as using their nose or tail to make contact, encircling females in a pattern like "numerical eight (8)", quivering against female bodies, and following the females by swimming [36,46,47]. While a different set of sexual behavior is found in females, which includes swimming alongside males, halting in front of males, approaching males by abruptly swimming towards them, and oviposition or laying eggs in their habitat. Zebrafish are early morning breeders, and they spawn in groups. Usually, the eggs of zebrafish are larger in diameter than other fishes (~ 0.7 mm) and they are translucent.

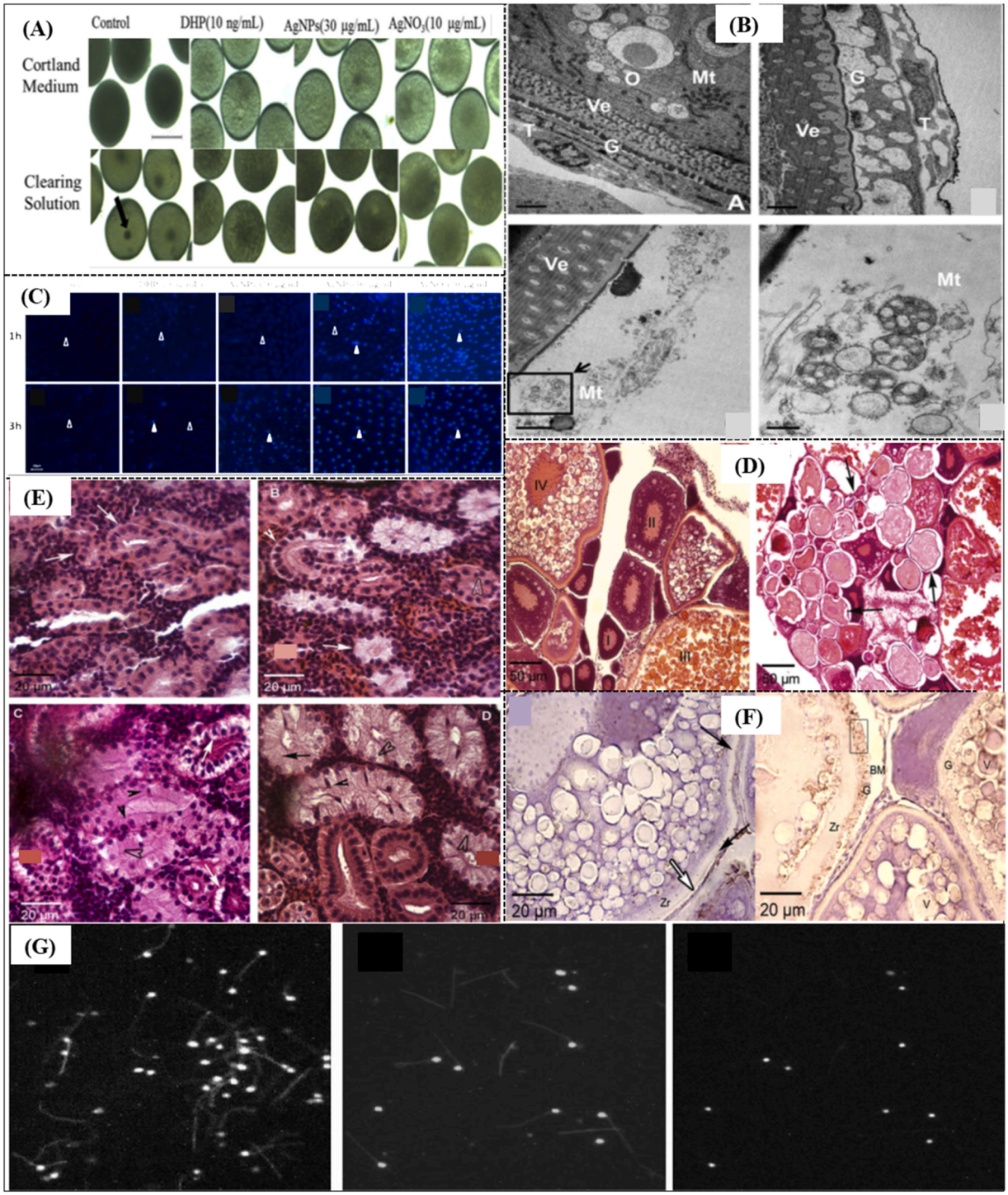

Fig. 3. (A) Silver nanoparticles (AgNPs) or $AgNO_3$ induce oocyte maturation in zebrafish. Ovarian follicles enclosing fully-grown immature oocytes remain opaque in the control group whereas they become transparent after the exposure of DHP, AgNPs or $AgNO_3$. After clearing solution treatment, germinal vesicles are observed at the center of oocytes in the control group, whereas they disappear after the treatment of DHP, AgNPs, $AgNO_3$ (H). The arrow indicates the germinal vesicle. Scale bar: 500 mm. (B) Effects of silver nanoparticles (AgNPs) or $AgNO_3$ on ultra structure of ovarian follicle cells. Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium containing designed reagents for 2 h. (A), control group showing normal appearance of ovarian follicle cells (Scale bar, 2 mm); (B) AgNPs (30 mg/mL) and (C) AgNO3 (10 mg/mL) treated group showing ovarian follicle cells have irregular cell morphology, acute vacuolation, nuclear condensation and fragmentation (Scale bar, 2 mm); (D) a high magnification image of the area indicated in C showing a novel mitochondrial swelling with intact inner mitochondrial membrane (Scale bar, 0.5 mm). G: granulosa, Mt: mitochondria, O: oocyte, T: thecal cell, Ve: vitelline envelope. [42].

3.2.2. Reproduction analysis

The remarkable reproductive efficiency of zebrafish confers additional benefits to the assessment of the effects of chemical agents on various aspects of reproduction, fertilization, and embryo viability. For instance, when zebrafish embryos are exposed to higher concentrations of TiO_2 nanoparticles, an increased mortality rate and reduced egg production is observed [48]. In contrast, exposure to silver nanoparticles shows a positive impact on embryos, such as enhanced maturation of

zebrafish oocytes as a result of increased oxidative stress and resultant follicle cell apoptosis [42]. Another study revealed that chronic exposure to ammonium perchlorate results in reduced spawn volume [49,50]. When bisphenol compounds are introduced excessively into embryos, they cause a skewed sex ratio that favors females, an imbalance of steroid hormones, a reduced count of germ cells, a decreased hatching rate, and embryonic malformation [51,52].

McAllister and Kime, in 2003 suggested a study, proposing the reproductive toxicity effects caused by tributyltin [53]. Pharmaceutical drugs, in addition to chemicals or environmental agents, can have detrimental effects on various reproductive parameters. These include the quantity of spawned eggs, success in hatching, gene transcription, hormonal levels, and histological alterations in the gonads [36,54,55]. The zebrafish model has also been preferred to assess the embryotoxicity of various drugs and hormones. In addition to this, experiments involving endogenous molecules have demonstrated their efficacy in predicting human drug safety [56–58].

3.3. Acute toxicity

Acute toxicity appraisal is the evaluation and assessment of potentially hazardous test substances and entails an analysis of the consequences caused by one dosage of the test substance. A better description of the acute toxicity testing is offered by the LD_{50} (=median lethal dose) and the LC_{50} (=Lethal Concentration 50) values derived from the toxicity experiment for a specific compound; the LD_{50} value signifies the dose which kills 50% of animals and the LC_{50} value describes the lethal concentration which kills 50% of animals. The salient features of acute toxicity testing include (a) identification of the target organ (b) Provides safety measures to the individuals engaged in the improvement and testing of biochemical substances (c) selecting appropriate doses for prolonged toxicity studies (d) The data generation comprises the detrimental effects of a biochemical substance on humans, animal well-being, and the environment (d) It offers the underlying basis for the design of other testing programs.

The evaluation of acute toxicity employs various methods, which include: (a) Miller and Tainter's graphical method (b) Reed and Muench's arithmetical method (c) Arithmetical method of Karber and (d) Lorke's method.

Miller and Tainter's graphical method: This approach is employed to determine ED50 values. It entails administering varying doses of the test substance to different groups, each maintaining an equal volume. The animals are categorized into five groups, with each group comprising ten animals. A dissolved test substance is used for group one animals while the remaining groups are administered varying doses of the test substance. In this technique, logarithmic doses are plotted on a graph against probits of the percentage [2].

Arithmetical method of Reed and Muench: This method incorporates a cumulative analysis of values derived from the study's results. The general assumption is that the mortality of animals can be attributed to the administration of higher doses of the test substance. The cumulative dead and survivors are documented. The survival percentage is calculated, followed by the computation of the LD50 [2].

Karber's Method: In this method various groups consisting of five animals each are treated with different doses. In each group, the animals are treated with particular doses; from group to group the number of doses increases in ascending order (the sequence begins with group 2, which receives the lowest dose). In this method, key parameters include the average number of recorded mortalities within each group and the difference in dosage across the groups. The determination of the lethal dose is carried out using the arithmetic method of Karber, outlined as follows [59]:

$$LD_{50} = LD_{100} - \Sigma\,[(a \times b) \div n]$$

Where LD_{50} = median lethal dose, LD_{100} = least dose required to kill 100%, a= does differ, b= mean mortality, n = group population.

Lorke's method: This method of toxicity testing consists of two phases: **Phase I:** During this phase, the division of nine mice were done in three groups, with each group comprising three mice. Each group is treated with the test substance concentrations of (10, 100, and 1000) mg/kg. At regular intervals, the observations are made after the groups are treated with the test substance. This is performed for the inspection and checking of the adverse effect of the test substance treatment (for example time to recover or time to death after the treatment). In this phase, the 24 hrs of the observational period is performed. **Phase II:** Depending upon the result obtained from Phase I, the stepping up or down of the doses is made in this phase and like that of Phase I, this phase also includes the division of three animals into three groups. In this phase, the animals are treated with a higher concentration of doses such as (1600, 2900, and 5000) mg/kg. Unlike Phase I, the toxic symptoms are not only observed for 24 h but also for 7-14 days. The calculation of the lethal dose is carried out using the formula provided below:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Where, D_0 = highest dose that gave no mortality, D_{100} = lowest dose that produced mortality.

3.4. Cancer-based investigations

Cancer has been widely recognized clinically and pathologically in almost all vertebrates and hence the need for vertebrate model systems has emerged to understand it for human [60]. It is not practically possible to fully capture the heterogeneous behavior and functionality of cancer using a single model system, thus we must rely on the uniqueness of different systems to further understand their complexity. While the mouse model is widely acknowledged for its efficacy in cancer research, the uniqueness and significant features of zebrafish have shed light on its potential for advancing in vivo cancer research [61].

3.4.1. Zebrafish as a model system for cancer research

Zebrafish have emerged as a valuable tool for studying cancer due to several reasons, such as their ability to develop fish lines with oncogenic transgenes and their potential for conducting genetic and pharmacological experiments. It has paved a new way to dissect mechanisms such as cancer formation, metastasis, malignancy, and angiogenesis. Apart from all these, zebrafish require minimum maintenance and care in the laboratory, and relative to the mice model, they are cost-effective as well [60,62–65]. Beyond this comparative genetics, zebrafish has many advantages large-scale multiplication, rapidly developing embryos, transparent structure, similar sequences with humans, etc. These characteristics facilitate convenient targeting of highly conserved cancer pathways through forward genetic screens [66]. Additionally, zebrafish provides additional advantages over cellular-based screening techniques through an in-vivo screening maintaining the quality of the tested libraries. Furthermore, using the entire organism is advantageous in forwarding phenotype-based screenings as it allows small molecules to interact with all biological pathways, rather than being limited to a subset of pathways within a specific cell line [67]. Similarly, utilizing a whole organism for screening various compounds enables the expression of diverse phenotypes resulting from multiple pathways, cell-niche interactions, cell-cell interactions, etc. Due to the *ex vivo* development of embryos, a more enhanced visual assessment of the observed phenotypic changes during chemical screening becomes possible.

3.4.2. Chemical Mutagenesis/ Treatment with mutagens

Zebrafish model has been used to understand the chemical carcinogenesis for several chemical compounds, which are carcinogenic to

mammals and are observed for adverse effects on the embryos [60,64,65,68,69]. The investigations appreciate water-soluble carcinogens for the ease of treatment because chemicals can be simply dissolved in water, allowing an extended duration of exposure. When zebrafish embryos were exposed to 7,12-dimethylbenz(a)anthracene, *N*-nitroso dimethylamine, and *N*-nitrosodiethylamine, tumors in the liver and intestine were kept under observation. A recent study showed that extensive exposure to *N*-nitrosodiethylamine leads to the development of liver and pancreas carcinomas [70] while *N*-nitroso dimethylamine specifically induces liver tumors [71]. Additionally, 7,12-dimethylbenz [*a*]anthracene induces a vast tumor spectrum which includes liver neoplasms, epithelial tumors in the pancreas, thyroid, intestine, mesenchymal tumors in cartilage, blood vessels, muscles, and neural tumors [72]. Other studies have indicated that chemicals like ethylnitrosourea, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, and diethylnitrosamine induce a wide range of tumors in zebrafish. Most commonly, hepatic tumors are observed but other organs like skin, muscle, and pancreas, might get affected as well [68].

Chemical carcinogenesis is advantageous in the study of tumor biology because of its simplicity and cost-effectiveness. The occurrence of spontaneous tumor initiation is observed within the appropriate tissue microenvironment, simplifying the studies considerably. The drawbacks include late tumor onset, low incidence of tumor development (B10%), and heterogenic development of tumors concerning background and location. Another limitation is the detection of tumors which requires gross visible appearance or histological evaluation of fixed zebrafish tissues. However, recent research has uncovered potential solutions to these limitations by establishing chemically-induced zebrafish tumor cell lines that can be cultured and transplanted into syngeneic animals [70].

3.4.3. Tumor Transplantation and in-vivo imaging

Tumor cell transplantation is one of the core methods being used in the assessment of tumors in model systems, wherein a donor donates a tumor cell to a recipient, either within the same species (allograft) or across different species (xenograft). In this technique immunocompromised mice such as nu/nu, non-obese diabetic (NOD), and severe combined immunodeficient (SCID) are transplanted with human cells. A significant drawback of this technique is the restriction on the number of animals that can be used, as grafting more than 10 to 12 recipients per group is impractical. Also, the imaging of the transplanted cells, typically achieved through the utilization of Green Fluorescent Proteins (GFP) or luminescence, is subject to certain limitations in terms of resolution [61].

Detailed transplantation studies are more effective for vasculature remodeling, cancer invasion, and metastasis. When the transplantation of human tumor cell lines is done to a zebrafish embryo, they exhibit the expression of fibroblast growth factor or vascular endothelial growth factor, leading to neovascularization within the tumor graft. However, this effect can be suppressed by the administration of antiangiogenic chemicals [73]. In another distinctive study, fluorescently labeled human breast cancer cells were injected into a one-month-old zebrafish embryo, which resulted in the expression of vascular endothelial growth factor (VEGF). The expression of RhoC stimulates vascular endothelial growth factor (VEGF), which, in turn, induces openings in vessel walls, enabling invasion [74]. Apart from xenograft transplantation, several transgenic and mutant cell lines have been utilized to observe the effects of carcinogens on different genes present in zebrafish. A few of those models used in-vivo techniques are; (a) Transgenic lines- Myc and TEL-AML1 leukemia models (b) MYCN and kRASG12D solid tumors models (c) kRASG12D embryonic rhabdomyosarcoma model (d) BRAFV600E melanoma model. As the zebrafish model continues to prove its potential as a valuable vertebrate model for studying tumor biology, it will be intriguing to witness the evolutionary advancements of zebrafish [68].

3.5. Neurobehavioral toxicity

Exposure to neurotoxicants may lead to significant risks in human health as they cause susceptibility in the blood-brain-barrier (BBB) system and the central nervous system (CNS) (Table 1). The neurotoxicity assessments have garnered additional attention when it comes to early-life organisms or newborns. In humans, the critical development of Blood-Brain-Barrier (BBB) occurs until 2 years of age whereas the BBB of zebrafish develops by 48 - 72 hpf. Claudin-5 and Zona occludens protein 1 (ZO-1) are the two proteins expressed in the tight junction of BBB of humans, zebrafish, and mice [75]. As depicted in Fig. 4, the neurotoxins may pass through the immature BBB via Claudin-5 and ZO-1 which may further cause damage to the brain. The developmental period, including prenatal and postnatal stages, may face adverse effects due to neurotoxic chemical compounds. Progress in neurotoxicity studies is important to avoid different neuropsychiatric circumstances like autism spectrum disorders [76], Parkinson's disease [77], and attention deficit hyperactivity disorders [78]. Developmental neurotoxicity testing (DNT) must go through numerous challenges when it is performed on a complex system like mammals. In such a situation zebrafish contributes to being an alternative non-mammalian model for the neurotoxicity assessment. The developmental brain structure in zebrafish shares analogous counterparts to the developing mammalian brain [79]. Due to the prolific nature and tiny size of zebrafish, the DNT can be performed in 96-well plates and it offers several significant advantages for DNT [80]. Habenula and amygdala are the structural regions of the zebrafish brain, where neurobehavioral studies have shown the presence of similar structural regions in humans [14]. Apart from these many brain subdivisions such as the cerebellum, cortex, thalamus, and hippocampus are identifiable both in mammalian and zebrafish larval brains [80,81]. The process of neural plate formation and neurulation exhibit similarities to the corresponding general processes observed in other vertebrates. Additionally, the development of neuroectoderm with neurogenesis in the zebrafish is found similar to that of other vertebrates [82].

The studies have amply demonstrated the reasons behind the similarities in the mammalian and zebrafish nervous systems. The vital body parts like the eyes (ocular system) and the ears (oto system) are connected to the brain via the Cranial Nervous System. Henceforth progress related to neurotoxicity assessments becomes a major concern to reduce such a higher mortality rate. The brain function, nervous system, and visual pathways play a key role in the locomotion behavior of zebrafish [17].

3.5.1. Gene expression profiling-based assessments for neurotoxicity studies

More than 80% of the conserved pathways related to human diseases overlap with zebrafish, and approximately 70% of the zebrafish genome demonstrates homology to the human genome [90]. Such a high rate of genomic similarities carves a pathway for the comparative study between zebrafish and mammalian gene expression mechanisms. The development and utilization of compatible genetic markers are key factors for performing assessments related to gene expression in zebrafish. At sub-toxic concentrations, the change in gene expression can be perceived in zebrafish without any visible changes in the phenotype [80]. Thus, the quantification of markers related to developmental toxicity can help in the faster detection of changes in gene expression [18]. Concerning the central nervous system, the zebrafish and the mammalian models share similar gene expressions. Transcriptional modification has also been observed in response to neurotoxicants [79]. For instance, the expression of the mammalian gene *achaete-scute* is the homolog for the *zash1a (ascl1a)* and *Mash1* genes present in the zebrafish and mouse model [91]. These types of homologous genes support the neurotoxicity assessments based on gene profiling studies. Multiple investigations related to gene expression have been performed on zebrafish using different chemical compounds. The study examined the gene expression of embryonic zebrafish exposed to ethanol, focusing on nervous system genes as potential markers of neurotoxicity [92].

Table 1
Chemical compounds responsible for several neurotoxic effects. (*No noticeable effects were observed).

Sl no.	Chemical Compound	Ototoxicity (ear)	Ocular toxicity (eyepiece)	Locomotory Effects	Effects on CNS	Reference
1	Retinoic Acid	*	*	Abnormal pectoral finbudmorphology and ectopicshh expression & Pectoralfinduplications.	Abnormaldevelopmentofthecaudalmidbrainandanteriorhindbrain.	[83]
2	Cyclopamine	*	Role of hedgehog signaling in eyedevelopment.	Inhibition offinoutgrowth.	Elimination of primary motor neurons.	
3	17-betaestradiol, diethylstilbestrol.	*	*	*	Effectsonmortalityandhatching,consequencesforCNS.	
4	Saxitoxin	*	*	*	Sensory, motor neuron defects.	
5	Arsenic (As)	*	*	Delayed behavioral development, stimulus, responses.	Altered axon outgrowth in the brain and nerve growth in the spinal cord.	[75]
6	Lead (Pb)	*	*	*	Altered global expression of genes related to neurological development and functioning.	
7	Organophosphorus pesticideChlorpyrifos (CPF)	*	*	Anomalies in swimming ability.	Inhibition of the axonal growth in primary motor neurons (PMNs) and secondary motor neurons (SMNs).	[18]
8	Teratogenic drugs	Delayed development of ear lobes.	Single eye (Cyclopia). Eyes absent. Uneven eyes. Abnormal shape of the eyes.	Kink in the tail. Tip of the tail uplifted/down. Bent tail—Tail flexed laterally or dorsoventrally.	*	[84]
9	Cadmium	*	*	Abnormal somite patterning and defectsinaxonogenesis.	Ectopicapoptosisinduction.	[85]
10	Copperandzinc	*	Delayed development of pigments.	*	Effects on hatchingandsurvival.	[86]
11	Lead (Pb)	*	*	Altered swimming activity under light or dark condition. Altered spontaneous swimming activity.	*	[87]
12	Methylmercury (MeHg)	*	*	*	Oxidative stress response, DNA repair mechanism	[88]

Potential markers for neurotoxicity have been identified by analyzing gene transcripts expressed in neuronal stem cells and/or developing neurons. On exposure to ethanol along with the alteration (increase or decrease) in the transcript of genes, considerable overexpression of a specific astrocyte marker was observed. For astrocytes and oligodendrocytes, *gfap* and *mbp* are the respective gene markers used. In another study of ethanol exposure, 1 to 6 dpf zebrafish were exposed to 1% of ethanol, and significant induction of *gfap* was observed [93,94]. Investigation of the neurotoxicity induced due to an organophosphate ester, known as triphenyl phosphate, has been performed using gene expression related to neurodevelopment; downregulation of *1-tubulin*, *mbp*, *syn2a*, *shha*, and *elavl3* genes showed the neurotoxic effects of triphenyl phosphate [95]. In an experiment, the assessment related to the effects of ibuprofen, diclofenac, and paracetamol was performed. The results showed the downregulation of the *neurog1* expression caused as a result of ibuprofen and diclofenac by 19% and 26%, whereas only ibuprofen up-regulated *neurod1* [96]. Another investigation revealed that exposure to low-dose of arsenite was responsible for disturbance in the defense pathway of zebrafish embryos [97]. Additionally, when zebrafish were exposed to methyl mercury at certain concentrations, the external malfunctions were not observed but it caused a disturbance in homeostasis [88]. In an experiment, zebrafish embryos were exposed to 11 toxicants, including lead, mercury, arsenite, cadmium, polychlorinated biphenyl, dioxin, and valproic acid. Interestingly, barcode-like toxic genomic responses were formed due to the 11 toxicants at specific concentrations without any morphological defects, [98]. Another instance of DNT is the neurodevelopmental gene expression of *mbp*, *syn2a*, and α 1-tubulin in larval zebrafish which was down-regulated on treatment with Tris (1,3-dichloro-2-propyl) phosphate and chlorpyrifos (CPF) [99]. These studies manifest that gene expression profiling in response to neurotoxic compounds is an important tool for neurotoxicity assessments.

3.5.2. *Neurobehavioral-based assessments for neurotoxicity analysis*

The neurobehavioral assessment involves the study of changes in locomotory behavior like the number of movements, response to touch activity (spontaneously occurring or elicited through stimulation), and swimming activity. Several critical observations in human locomotion show the importance of neurobehavioral assessments. For example, movement disorders were observed in humans due to anti-depressants and antipsychotic drugs [27]. The zebrafish model helps in easy tracking of the in vivo behavioral changes triggered by any chemical compounds. Additionally, the neurobehavioral changes in the zebrafish larva can be tracked in 96 well plates with the help of an automated video-tracking system that is commercially accessible [100].

Several studies have uncovered results signifying the neurotoxic effect of substances on the behavior of motion in zebrafish which have also been proven harmful to human health. The locomotory study in zebrafish involves the quantification of parameters like spontaneous tail coiling, swimming speeds, distance moved, and turning rates. For instance, a substantial decline in the locomotor activity of larval zebrafish was uncovered when it was treated with an environmental toxicant named, triphenyl phosphate [95]. Another study was performed on 1–5 dpf zebrafish which was exposed to 60 water-soluble compounds and the experiment resulted in locomotion suppression (monotonic concentration-response), stimulation (monotonic response), and stimulation followed by suppression (biphasic response) [101]. A study showed that the number of tail coilings got decreased when zebrafish were exposed to mercury [102]. Moreover, it was revealed that various pyrethroid insecticides led to an increment in the motility rate of

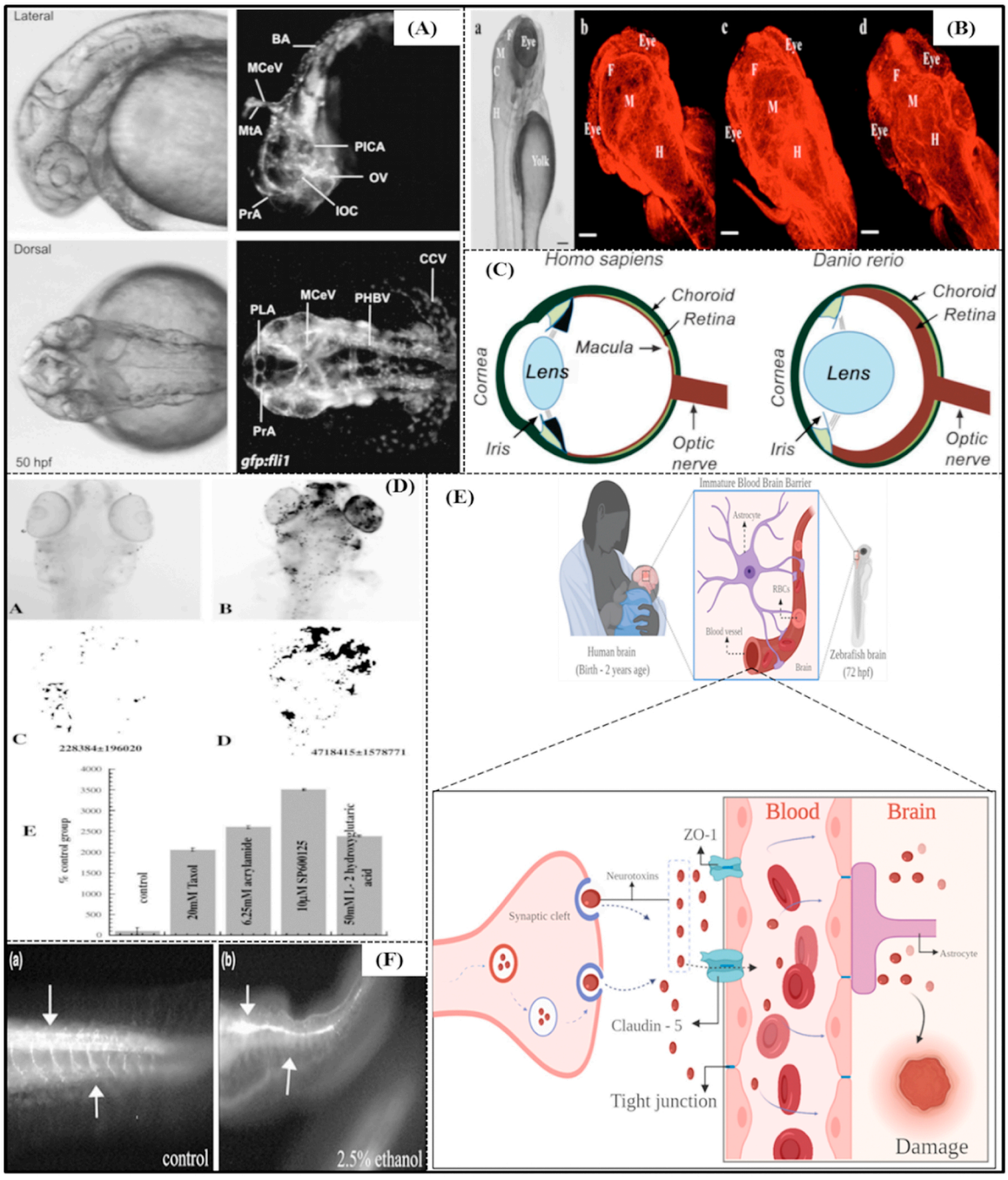

Fig. 4. (A) Visualization of the zebrafish embryo vasculature by gfp:fli1 expressing endothelial cells. Light and fluorescence microscopy of transgenic zebrafish embryos at 50 hfp in lateral and dorsal orientation. BA: basilar artery, CCV: common cardinal vein, IOC: inner optic circle, MCeV: middle cerebral vein, MtA: mesencephalic artery, OV: optic vein, PHBV: primordial hindbrain channel, PLA: palatocerebral artery, and PrA: prosencephalic artery [83]. (B) Zebrafish neurodevelopment. (a) At 72 h post fertilization (hpf) major subdivisions of the zebrafish brain are present; Zebrafish axonal networks visualized by acetylated α-tubulin staining (b) at 72 hpf, (c) at 96 hpf, and (d) at 120 hpf of development. Scale bar = 100 μm. (C, cerebellum; H, hindbrain; M, midbrain; F, forebrain) [75]. (C) Comparison of the human and zebrafish eye: Human and zebrafish eyes mainly differ in lens shape and space between the lens and retina [89]. (D) Apoptosis assessment in whole animals. TUNEL staining was performed to detect apoptosis (black dots) in zebrafish after taxol treatment. Dorsal views of the brain are shown. Untreated control zebrafish showed low level of brain apoptosis, consistent with developmental stage (A). Taxol-treated zebrafish showed abnormal apoptosis in both the brain region and the spinal cord (B). Morph metric analysis was performed to quantify staining signals. As shown in (C, D), staining signals were 228,384 ± 196, 020 and 4718,415 ± 1578,771 for control and taxol-treated zebrafish. Apoptotic level of zebrafish after taxol, acrylamide, SP600125 and L-2 hydroxyglutaric acid treatment were examined using TUNEL staining and morphometric analysis (E). A significant increase in apoptosis was observed after taxol, acrylamide, and SP600125 and L-2 hydroxyglutaric acid treatment. (E) Neurotoxins released into the brain via the immature blood-brain barrier (BBB) and ultimately causing damage in the brain [75]. (F) Zebrafish motor neuron damage after ethanol treatment. Forty-eight hours post fertilization zebrafish were untreated (a) or treated with 2.5% ethanol (b). Anti Znp1 antibody was used to visualize motor neurons in the tail region (anterior: left; posterior: right) and ethanol treatment resulted in motor neuron loss [16].

larval zebrafish [103–105]. Locomotion assay related to antipsychotic drugs has also been performed in zebrafish which involved antipsychotic drugs such as fluphenazine, olanzapine, and haloperidol. The experiment resulted in a reduction in swimming speed caused due to all three drugs; compared to other antipsychotics, the effect of olanzapine was found to be extensively milder. The effect of drugs on the zebrafish dopamine pathway was a notable observation that was made from this experiment since the same observation was made in human beings. The characterization of effects due to endosulfan and endosulfan I in zebrafish larvae was performed wherein a reduction in touch response and some cases paralysis was observed in the larvae which were treated with acute doses [106]. Performing various assays related to the screening of locomotory changes has become a vital part of studies. The larval photo motor response assay was performed for the assessment of benzo[*a*]pyren since tracking of movements over alternating light and dark periods were permitted by this assay; significant hyperactivity was caused due to the high dosage (4 mM) of benzo[*a*]pyrene [107]. Henceforth, the neurotoxicity assessment can be improved by neurobehavioral studies as it has become one of the major assets for the improvement in neurotoxicity studies of various substances.

3.6. Ocular toxicity assessment

The ocular system relates to the nervous system and the study pertaining to analyses of substances behind such a harmful cause has become an important factor. Studies have shown similarities in the zebrafish and human ocular anatomy. Important parts of the eyes such as the lens, retina, cornea, innervation, and vascularization are similar in humans and zebrafish [89,108]; highly conserved gene expression is found in zebrafish and human eyes [109]. These significant factors make the zebrafish model a perfect system to study ocular toxicity. As per morphological, electrophysiological, and behavioral criteria the zebrafish vision is well developed by 4 dpf - 5 dpf [17,80].

The optokinetic response (OKR) and optomotor response (OMR) are the standardized assays that have been developed for measuring the functions of the larval zebrafish eye. The OMR assay +is performed for the measurement of locomotion in response to visual cues. In the OKR assay, an immobile fish is exposed to vertical stripes that alternate between darkness and light. The eye saccades of the fish are then quantified as a measure of a healthy eye response to moving stimuli [17]. Although easier automation is an advantage of OMR assay, then again identification of compounds causing motor defects unrelated to the ocular system is a big disadvantage of this assessment [27]. An OMR assay was performed to determine the effects caused by 27 compounds in zebrafish visual function [110]. Among 27 tested compounds, 19 of them were known to affect human vision and 8 of them do not have any effect. The experiment came out with the result that 13 of the nineteen positive compounds were also found positive in the zebrafish (68% sensitivity). In an experiment, an OMR assessment was performed for nine compounds and seven compounds among them were correctly predicted in both humans and zebrafish [57]. The rest of the two compounds (atropine and lithium) lead to defects in the human visual system whereas no effects are shown in zebrafish since. This is so because atropine and lithium affect binocular vision and in zebrafish binocular vision is absent. Thus, some limitations exist in the detection of drug effects in the vision system (Fig. 4).

3.7. Ototoxicity assessment

The auditory systems of humans and zebrafish are thought to have a common evolutionary origin, setting them apart from the auditory systems of other vertebrates. Along with conserved genetic regulations the zebrafish and human ear systems share similar neuronal signaling, auditory anatomy, and homologous hair cell physiology. Additionally, studies performed on zebrafish have supported a large part of the human hearing and balance system [17]. The ear system of vertebrates consists of the inner, middle (eardrum and tympanic bones), outer (visible part) parts, and cochlea. Amplification of sounds in mammals is performed using the cochlea [111], but the primary organ responsible for both hearing and balance is predominantly the inner ear. In the zebrafish ear system, an outer ear, middle ear, and cochlea are absent, and it consists of only an inner ear which is like the vertebrates. The inner ear of zebrafish develops by 5dpf and it possesses neuromasts as mechanosensory receptors that exhibit sensitivity within a frequency range spanning from 100 to 5000 Hz [112]. A rosette of hair cells in a framework of supporting cells is present in each neuromast [113]. The mechanical forces are transformed into an electrical pulse by the hair cells and then the electrical pulse is carried to the brain by the auditory nerve [80]. In zebrafish, certain fundamental dyes are widely used for the structural evaluation of the lateral line hair cell [114]. For high-throughput screening assays, mostly fluorescent in vivo dyes are utilized to visualize the zebrafish hair cells [27]. The lateral line hair cells have vital features such as amenability for stains and dyes, clear-cut inference in rheotactic behavior, and enormous ease of access [115]. Thus, studying the effects on lateral line hair cells is a significant part of ototoxicity assessment.

Drugs such as platinum-based anti-cancer drugs, aminoglycoside antibiotics, anti-malaria, or nonsteroidal anti-inflammatory drugs are the reason behind human ototoxicity [116]. Experiments with zebrafish revealed neurotoxic effects such as deficits in behavioral responses caused due to metals for example copper, cadmium, and other hair cell death; the same experiments showed hair cell death caused by the same metals [117]. Cisplatin and neomycin were found to be ototoxic drugs since they were sensitive to the mammalian inner ear hair cells as well as lateral hair cells of zebrafish [118,119]. An ototoxicity assay was performed by Chiu et al. in which a library of 1040 compounds was evaluated in a zebrafish model [120]; most of the 1040 compounds were FDA-approved drugs and in the initial screening, 95 compounds were identified as ototoxic. Moreover, retesting of 21 compounds was responsible for ototoxicity. It was found that nine of the 21 compounds have ototoxic effects in humans but for the remaining twelve compounds no data existed. Further studies explained the toxic effect in mouse utricle explants caused by two of the novel ototoxic compounds. This experiment served as the confirmation of the zebrafish model for ototoxicity assay. Sound-evoked potential audiometry is another assay for the assessment of hearing abilities in fish. In this approach utilizing cutaneous electrodes, the field potentials elicited by an auditory stimulus are recorded [121]. Since the screening of ototoxicity in the mammalian system is rarely done as well as it is a tough task, henceforth, up to a certain extent zebrafish system shows to be an excellent model for ototoxicity-based assay. Future studies are required to monitor more compounds and study their mechanism of ototoxicity (if shown), as well as upsurge further ototoxicity-based assays.

3.8. Cardiotoxicity assessment for safety pharmacology studies

Heart is one of the first developing organ which start functioning in Zebrafish. The development takes place within 22 hpf. The development of the heart occurs from the lateral mesoderm in two bilaterally symmetric fields. By 48 hpf, the cardiovascular system becomes fully operational, showcasing a diverse range of ion channels and metabolic processes [122]. During the initial phases of Zebrafish development, the ERG *(ether-a-go-go-related gene)* is expressed. The human ether-a-go-go-related gene (hERG) encodes the pore-forming subunit of

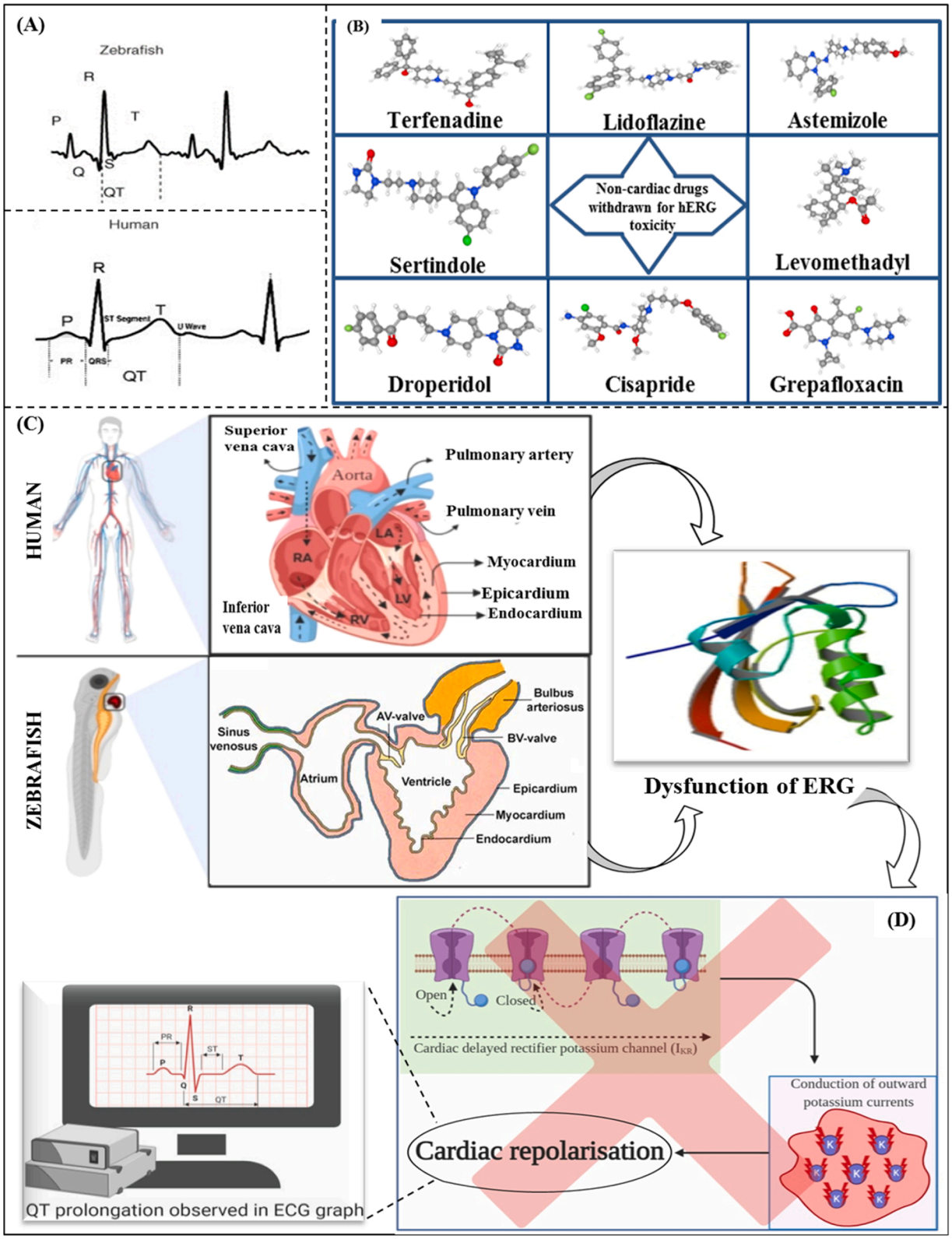

Fig. 5. **(A)** Comparison of human and zebrafish electrocardiograms (ECG). Humans and adult zebrafish exhibit similar ECG patterns [16]. **(B)**Non-cardiac drugs withdrawn for hERG toxicity [124]. **(C)** and **(D)** Dysfunction of the ether-a-go-go-related gene (ERG) in humans and zebrafish leading to a dilemma in cardiac repolarization (QT prolongation observed) [120,125].

the rapidly activating delayed rectifier potassium channel (I_{Kr}), which plays a crucial role in cardiac repolarization. Long QT syndrome and sudden death are caused due to the dysfunction of hERG (Fig. 5), which arises in patients suffering from cardiac - ischemia [123]. There is 99% of conservation between the amino acid sequence which is responsible for the formation of a pore in zebrafish ERG and human ERG. The heart functions of adult zebrafish and humans have a striking similarity, demonstrated by electrocardiogram (ECG) reports of humans and zebrafish (Fig. 5); (a) PR interval (activation of atrial potential and conduction to the ventricle), (b) QRS complex (activation of the ventricle), (c) QT interval (duration of the ventricular action potential).

Merck & Co. (an American pharmaceutical company) launched an antiarthritic drug known as Rofecoxib (Vioxx) which was later withdrawn from the market due of an increased risk of heart attack and stroke. Propulsid (Cisapride) is another example of an oral gastrointestinal prokinetic agent, but it has led to 400 adverse cardiac events (arrhythmia) and has caused 80 deaths in the USA. As a result of these findings, Propulsid along with terfenadine (an antihistamine) and grepafloxacin (an antibacterial) was removed from the US market [16] thus substantiating that drug withdrawal is a major problem caused due to unpredicted cardiotoxicity. Hence, to avoid this issue and for cardiotoxicity assessment of substances **(**Table 2**)**, different toxicological assessment becomes priority.

3.8.1. *QT Prolongation-based cardio-assay*

QT prolongation refers to an extension of the QT interval due to the delay in ventricular repolarization. QT prolongation raises the risk of a potentially life-threatening cardiac arrhythmia called torsade de Pointes (TdP). For investigating cardiovascular disease and drug-induced cardiac QT prolongation, zebrafish has emerged as an valuable model organism [132]. Cardiac currents analogous to other species has been observed in cultured 3 dpf zebrafish [133]. In both chambers of the embryonic heart, the KCNH2 homolog genes are expressed which are relevant to human genes KCNH2, also known as hERG (human ether-a-go-go-related gene), particularly found in the pore and QT drug-binding region. It has been found that bradycardia and a 2:1 atrioventricular block (i.e., the ventricle beats 50% as frequently as the atrium) are induced due to the inhibition of KCNH2 by using morpholino antisense oligonucleotides in zebrafish embryos [134,135]. Two mutant alleles of KCNH2 in zebrafish were identified and characterized by Arnaout et al. (Fig. 6) [136]. A phenotype reminiscent of mutation-positive long QT syndrome in humans is produced by both alleles. The presence of two mutant alleles resulted in significant issues such as heightened duration of cardiac action potential in embryonic hearts, an elongation of the QT interval in adult electrocardiograms (ECGs), and enhanced sensitivity to QT-prolonging drugs. Moreover, QT-prolonging drugs can enhance the corrected QT interval and this has been demonstrated by the ECG recordings in adult zebrafish although any significant change is not caused by the control drugs [137].

In humans, it has been uncovered that QT prolongation, cardiomyopathy, arrhythmia, and negative isotropic effects were caused by drugs like thioridazine, terfenadine, mitoxantrone, and clomipramine. Whereas in zebrafish, these drugs were responsible for bradycardia, slow circulation, abnormal atrial, and ventricular (AV) ratio, and decreased contractility. In a study involving the testing of one hundred small molecules, it was shown that out of 23 drugs 22 were accountable for QT prolongation in humans and resulted in blocked AV conduction, and instigated bradycardia in zebrafish [140]. Pentamidine was one of the compounds identified that have been reported to produce QT prolongation via inhibition of hERG trafficking to the cell surface instead of through the direct block of the hERG channel [141]. Focusing on other studies of cardiotoxicity assay, the effect of nine drugs on heart rate in 3 dpf zebrafish was examined by Berghmans et al. [57] and was divided into the following three sets -

Table 2
The effects of various drugs and chemical substances on cardiotoxicity and cardio protection studies performed on zebrafish embryos.

Sl No.	DRUG	Effects observed	Reference
1	Anti-cancer drugs like- Daunorubicin, Pirarubicin, Doxorubicin (DOX), Epirubicin, and DOX-liposome. (anthracyclines)	a) Incomplete looping of the heart tube, b) Pericardial edema and bradycardia. c) down regulates the genes and protein expression related to cardiac development.	[126]
2	Cardiotoxic drugs like- aspirin, clomipramine hydrochloride, cyclophosphamide, nimodipine, quinidine, terfenadine, and verapamil hydrochloride.	a) Pericardial edema, b) Circulation problem, hemorrhage, and thrombosis. c) Bradycardia.	
3	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (organic pollutant)	malformed heart, defective atrioventricular valves, and pericardial edema.	[127]
4	two aryl phosphate ester (APE) components, triphenyl phosphate (TPP) / mono-substituted isopropylated triaryl phosphate (mono-ITP)	cardiac defects producing a tube-like heart.	
5	Phenanthrene (environmental cardiotoxic agent)	pericardial edema, abnormal heart looping, and enlarged ventricle.	
6	Diterpene alkaloids (DAs) and three diester diterpene alkaloids (DDAs) like aconitine (AC), mesaconitine (MAC), hypaconitine (HAC)	Malformation of heart, pericardial effusion, atrioventricular arrhythmia, and heart arrest. Severe cardiotoxicity.	[128]
7	Chemical compounds- BAYK8644 and terfenadine.	Accumulates within the embryo and delays heart formation or cause malformation.	[129]
8	Flecainide, dofetilide, terfenadine, cisapride, quinidine. (drugs)	Displayed type 2:1 arrhythmia (associated with hERG inhibition).	
9	Verapamil, thioridazine, BAYK8644, and JNJ303. (drugs)	Displayed other adverse cardiac effects, salmeterol produced cardiotoxicity at 24 h treatment.	
10	Rofecoxib (Vioxx), Merck's blockbuster antiarthritic drug.	Increases the risk of heart attack and stroke.	[16]
11	Propulsid (Cisapride), an oral gastrointestinal prokinetic agent.	Causes cardiotoxicity and adverse cardiac events (arrhythmia). Fatal in higher doses.	
12	Terfenadine (antihistamine) and Grepafloxacin (antibacterial)	Bradycardia and cardiac arrest.	
13	Mitoxantrone, terfenadine, clomipramine and thioridazine.	Cardiomyopathy, arrhythmia, bradycardia, abnormal atrial, and ventricular (AV) ratio, decreased contractility, and slow circulation in zebrafish.	
14	Artesunate (ART)- anticancer, anti-inflammatory, antiparasite, antioxidant, and immunoregulatory effects.	Cardioprotection- exerts anticancer effect by inducing cell apoptosis, antagonizing angiogenesis, reversing immuno-suppression of tumor cells.	[130]
15	Erythromycin, N-Acetylprocainamide (NAPA), Pentamidine, Procainamide, Sotalol.	Causes QT prolongation or TdP but do not cause bradycardia or any ventricular blockage hence not toxic to the heart.	[131]

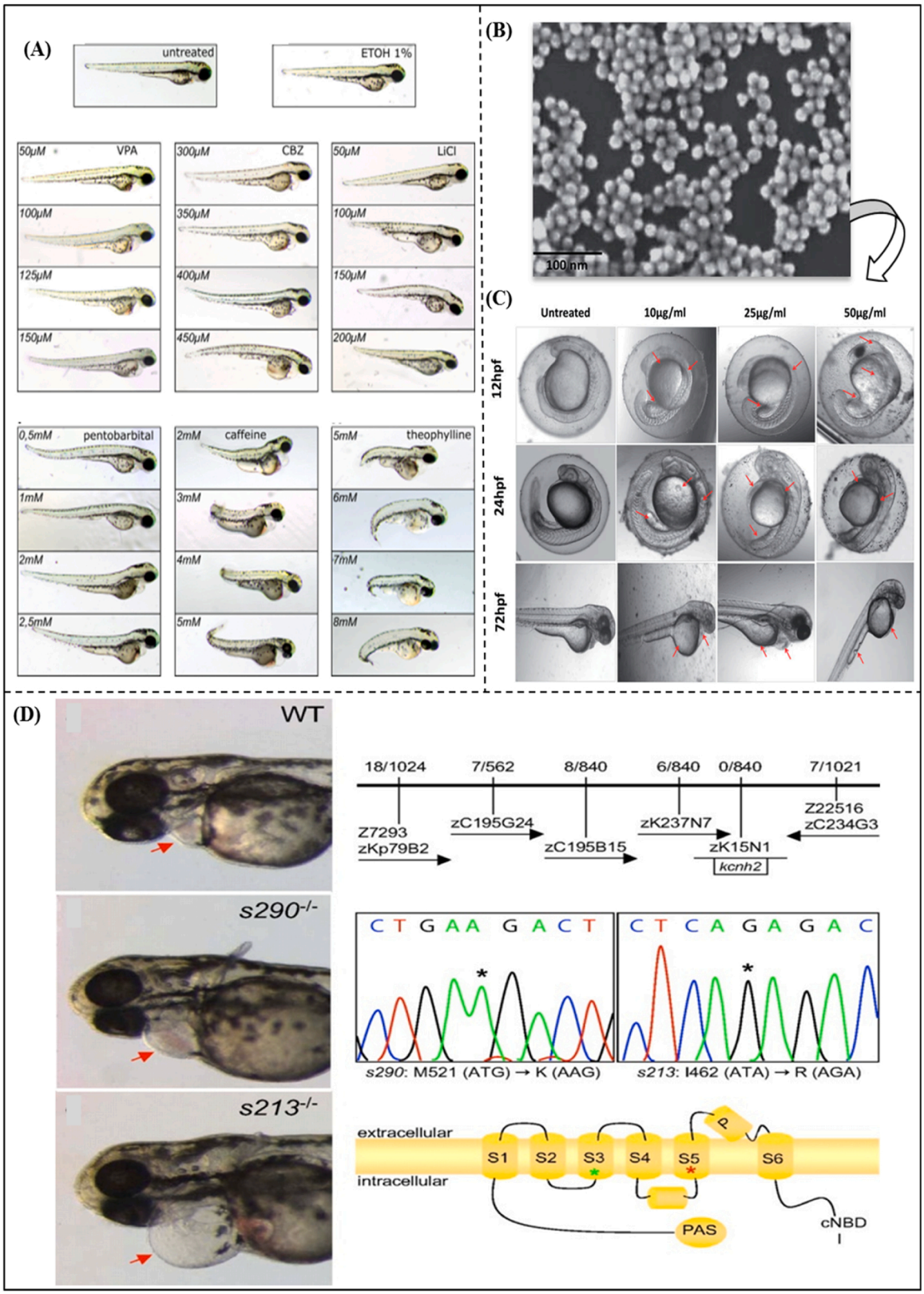

Fig. 6. (A) Morphological defects after 24 h of exposure. Representative images of 3 dpf larvae after 1 day exposure to psychotropic drugs at the indicated concentrations: untreated (A), ethanol 1% (B), VPA (C), CBZ (D), LiCl (E), pentobarbital (F), caffeine (G) and theophylline (H) [138]. (B) FSEM characterization of Au NP synthesized from *Calotropis gigentea,* and (C) Observation of swollen yolk, abnormal notochord development and pericardial edema in the bright field images of zebrafish embryos exposed to different concentration of green synthesized Au NP [139]. (D) Molecular analysis of two *kcnh2* mutant alleles [136].

The first set consisted of two drugs with unknown effects on cardiac function.
The second set comprised five known or reported hERG blockers.
The third set contained two drugs associated with tachycardia.

Zebrafish were used to screen the three sets of compounds mentioned above. The results indicated that from the first set, isoprenaline demonstrated an association with elevated heart rate in humans and induced mild tachycardia in zebrafish. Conversely, cromakalim exhibited no discernible effects. The second set led to the conclusion that among the five reported hERG blockers, four of them were responsible for severe bradycardia, and atrioventricular decoupling was caused by three of them. An unexpected observation was obtained when no effect on heart rate was produced even by 1 mM concentration of moxifloxacin whereas TdP and QT prolongation has been associated with moxifloxacin. In the last set, both negative compounds were found to have no impact on heart rate in the assay. These experiments and their outcomes show that reason behind apparent false negatives in zebrafish cardiac assays is the likely explanation is the poor absorption of the compounds. Henceforth, experiments and their data related to QT prolongation answered many questions related to the cardiac disorders caused due to some substances along with clarifying the similarity between human and zebrafish cardiac systems.

3.8.2. Source of cardiotoxicity by some clinical drugs

zebrafish can be commonly deployed for screening anti-cancer drugs. Identifying novel compounds such as endothelial or cardiomyocyte protective agents or antioxidants can contribute to the prevention of these adverse side effects [142]. Furthermore, by identifying high-risk patients several tragic situations can be minimized. The discovery of novel biomarkers helps us to deal with the identification processes. Anthracyclines (ANTs) are a class of drugs that are used as anticancer agents and other members of this class of drugs include Pirarubicin, Daunorubicin, Epirubicin, Doxorubicin, and DOX- liposome [126]. Some harmful events like pericardial epidemia, bradycardia, and incomplete looping of the heart tube were witnessed in embryonic zebrafish after they were exposed to various ANTs; the lowest amount of toxicity was produced by DOX [143]. Consequently, these studies revealed that ANTs downregulate genes and proteins associated with cardiac development. Most importantly, it was found that the cardiotoxic effects in zebrafish induced by ANTs were comparable to those reported in other mammalian models [127].

For further studies, several drugs such as 5-fluorouracil and mitoxantrone, DOX, Cyclophosphamide, and terfenadine from diverse categories were evaluated [144] including other categories of drugs such as cancer drugs, antiarrhythmic, beta-blockers, and anticonvulsants. The assessment of cardiotoxicity was based on parameters such as measurement of heart rate, circulation, heart rhythm, and morphological changes. From the test, similar results were obtained when 2 dpf embryonic zebrafish were treated with drug concentrations ranging from 0.01 μM to 1000 μM for 24 hrs; a major problem such as bradycardia was caused by DOX and Cyclophosphamide whereas pericardial edema and hemorrhage were induced by Terfenadine and Clomipramine. Interestingly, it was found that in humans, QT prolongation was led by Terfenadine and Clomipramine [145]. Gentamicin, Amantadine, and Tetracycline are some of the antibiotics and antiviral drugs that are used by people in their day-to-day life. These antiviral drugs rarely induce cardiotoxicity either in humans or in zebrafish and consequently, these drugs are used as a negative control in zebrafish cardiotoxicity assays [146]. Human cardiotoxic drugs such as aspirin, clomipramine hydrochloride, cyclophosphamide, nimodipine, quinidine, terfenadine, and verapamil hydrochloride were studied by Zhu, et al. in another experiment [147]. The administration of drugs was performed via soaking or yolk-sac microinjection. From this experiment, it was found that aspirin was responsible for the elevation in the heart rate whereas bradycardia was seen to rise by other drugs.

3.8.3. Nanoparticle-induced cardiotoxicity

The assessment of biocompatibility of nanoparticles in the systems is the most needed measure; given the use of nanoparticles in the drug delivery system. Various research on metal nanoparticles by Verma et al. offers us the major details regarding several nanoparticle-induced cardiotoxicities. For instance, an assessment of gold (Au) nanoparticles (NPs) was performed on (12 - 72 hpf) zebrafish model system [139]. The experiment involved commercial AuNP and green-synthesized AuNP via the floral extract of *Calotropis gigantea* (named CAuNP). Post-treatment showed a reduction in a heartbeat with an increase in the concentration of both AuNP and CAuNP, but a more pronounced reduction was noted in AuNP. Along with increased heartbeat, a significant result of pericardial edema was recorded in both cases. Another experiment was performed by Verma et al. in which the zebrafish embryos (24 - 72 hpf) were treated with magnesium oxide (MgO) nanoparticles (NPs) [148]; the problem of pericardial edema at higher concentrations of 250 μg/mL and 500 μg/mL was discerned.

Silica nanoparticles captivated a great interest in the healthcare sector due to their variable applications. One of the experiments by J. Duan et al. involved the assessment of cardiotoxicity induced by silica nanoparticles in zebrafish [149] wherein embryonic zebrafish (24 - 96 hpf) were exposed to silica nanoparticles. On the measurement of heart rate at 24 and 48 hpf, the report revealed remarkable bradycardia at the concentrations of 100 and 200 -μg/mL. Assessment of cardiotoxic effects produced due to silica NPs via western blot analysis of the cardiovascular proteins was performed as well. The results of the western blot came up with no important alteration in vascular endothelial growth factor receptor 2 (VEGFR2), ERK 1/2, MEF2C/ NKX2.5, and neither in β-actin. A contrary result was obtained, in a dose-dependent manner, which showed inhibition in the expression of angiogenesis-related ERK1/2 and phosphorylated VEGFR2 thus directing attention to the lethal cardiotoxic effects of some nanoparticles.

The above experiments lead to the various approaches of cardiotoxicity-based assessments along with their informative results. QT prolongation, pericardial edema, bradycardia, etc. were found to be some common trouble-causing diseases by drugs and some nanoparticles. Black peppercorns are a vital food product in our day-to-day life. A recent investigation by Patel et al. reported the biocompatibility of black peppercorns using the zebrafish model [150]. Where, along with heart deformity, the deformation in the chorion, yolk sac, tail, and notochord was also observed in the study. As a preclinical requirement, the exposure of any chemical substances for their benefit to nature and mankind must be verified before releasing it to the masses. Comparable cardiotoxic effects of substances in both humans and zebrafish can be observed. For exposure to more toxic substances, further investigations ought to be required on the pathways behind the genes/ proteins responsible for cardiotoxicity. Therefore, for the cardiotoxicity assessment, the observations guide the justification of zebrafish as an admirable model organism.

3.9. Hepatotoxicity assessment

Toxicity analysis of the organ responsible for the removal of toxins is imperative. Vertebrates carry many crucial organs among which the liver aids in the synthesis of proteins which help in the detoxification of harmful substances from the body. According to a study, it was found that the zebrafish and human liver share similar cellular counterparts apart from the Kupffer cells and the resident macrophages of the liver [151]. In order to facilitate the normal development of the liver in zebrafish, it is crucial to maintain the endoderm properly as the zebrafish liver originates from the anterior foregut endoderm [152]. Investigations have confirmed that signaling pathways such as Nodal, BMPs, and transcription factors like Gata4–6, Foxa factors, and Sox 32/17 play a vital role in zebrafish and mammalian endoderm development [153,154] [155]. Similar mechanisms of oxidative stress and the induction of enzymes against xenobiotic chemicals are carried out in

Table 3
Chemical compounds responsible for Hepatotoxicity and their potential effects in the liver.

Sl No.	Chemical compound	Target organ	Effects observed	Reference
1	Tricyclazole (pesticide)	Liver	Severe results for vertebrate hepatic development and function.	[157]
2	Triptolide (diterpenoid epoxide), a drug.	Liver (induces hepatoxicity in the zebrafish larva)	Hepatocyte vacuolation, disarray, oncotic necrosis, and a reduction in liver volume.	[158]
3	N-ethyl-Nnitrosourea (ENU)	Identified essential genes and gave insight into the physiology of liver development and function.	Effects of liver development by causing mutations in larval stages.	[159]
4	S-glutathione reductase (GSNOR), an enzyme	liver regrowth after partial hepatectomy.	Chemical inhibition of GSNOR accelerated hepatic regrowth.	
5	Acetaminophen (APAP)	Liver	Overdose of this drug is the leading cause of drug-induced acute **liver failure**.	
6	Nitroreductase (NTR) an enzyme	Liver	Catalyzes the reduction of the prodrug metrodinazole (Mtz), thereby producing a cytotoxic product that induces cell death.	
7	Acetaminophen, a commonly used drug.	Liver and other organs.	Induces hepatotoxicity as side effects.	[160]
8	Amiodarone, a drug.	Liver and other organs.	Amiodarone induces embryo lethality at 10 μM and is potentially hepatotoxic.	
9	Isoniazid (INH) - a first-line antituberculosis drug.	Liver as well as other organs.	INH 37 showed increased expression levels of ERS-related factors, autophagy-related factors, and apoptosis-related factors in larvae.	[161]
10	Cytosine arabinoside	Liver, heart, GI, etc.	Potentially hepatotoxic.	[16]

humans and zebrafish [16,156]. These major similarities are shreds of evidence that the hepatotoxicity assessments in zebrafish may lead to new findings in support of people dealing with liver problems. In both the cases, of human beings and zebrafish, the regenerative capacity of the liver as compared to other organs is remarkable. In an effort to reduce the expenses and time associated with toxicity assessments, numerous hepatotoxicity test systems have been devised. Testing categories like serum enzyme tests, hepatic excretory tests, assessments of alteration in the chemical constituents of the liver, and histological analysis are required for the evaluation of drug-induced hepatotoxicity [16]. The use of a human hepatoma-derived HepG2 cell line is one of them and is commonly deployed in hepatotoxicity assay [17]. To acquire a detailed view regarding these studies, numerous significant assessments related to hepatotoxicity are discussed further **(**Table 3**).**

3.9.1. Assessment of drug-Induced Liver Injury (DILI) using Zebrafish as a model

Clinical research and the biomedical fields need to go through several challenging circumstances during the discovery and development of a drug. In some critical situations, the patients suffer from grievances caused due to wrong medical drugs treatment. For example, Acetaminophen and paracetamol are consumed by more than 60 million population of America and are used in combination with opioids and diphenhydramine. Using higher concentrations of this drug at a level of 7.5 g/day results in increased levels of toxicity [162]. These circumstances lead to the assessments of Drug-Induced Liver Injury (DILI).

Cytochrome P450 (CYP) assay is one of the fundamental enzymatic assays performed for the hepatotoxicity assessments of drugs. The CYP enzymes are located in the liver and are responsible for certain important metabolism. The activation and inactivation of several endogenous and exogenous compounds are regulated by oxidative catalytic transformation [163]. The reactions of CYP enzymes occur in two phases namely Phase I and Phase II. Crucial events like oxidization, reduction, or hydrolyzation of the metabolized compound take place in phase I, and these reactions are mediated by the CYP enzymes while they do not mediate the process of conjugation occurring in phase II [164]. Most of the drug-metabolizing reactions are catalyzed by CYP3A4 and CYP2D6 enzymes. The stimulation or/and inhibition of these two CYP enzymes are linked with many clinically relevant drug-drug interactions [16, 165]. The study of Ibuprofen which is an anti-inflammatory non-steroidal drug shows the analogy between the CYP of humans and zebrafish. The metabolization of this compound occurs via different reactions like the oxidation of the parent compound to hydroxyl-ibuprofen and carboxyibuprofen along with the conjugation of glucuronic acid of both, the metabolite and parent compounds [166]. The oxidation of ibuprofen in humans is catalyzed by the CYP2C8/9 isoform [167]. Hydroxylated ibuprofen in the zebrafish extracts and water samples has been detected after the exposure of ibuprofen to embryonic zebrafish [168]. This observation shows the resemblance between human and zebrafish metabolic systems; a recent study also indicates that human CYP3A4 is an orthologue for the zebrafish CYP3A65 [169]. As mentioned earlier the investigation of toxic effects produced by higher doses of paracetamol also becomes important. Interestingly, scientists also found that human CYP3A4 and zebrafish CYP3A65 are accountable for the hydroxylation of testosterone in phase I along with the formation of NAPQI [163,170]. Additionally, native enzymes such as biotin and carboxylase are present in the liver and gut of zebrafish. The measurement of these enzymes can be performed using the reporter enzyme assay. One of the studies in zebrafish showed the hepatotoxicity effect of isoniazid (INH) which is a first-line antituberculosis drug. The study conveyed that INH was responsible for increment in the ROS levels which ultimately led to oxidative stress [161]. These important mechanisms support the fact that as compared to human beings, the zebrafish model for the assessments is related to DILI.

4. Why Zebrafish model for toxicity-based assessments?

Danio rerio commonly known as Zebrafish is emerging as a lucrative model for answering elemental biological questions. The swift increase in publication integer employing zebrafish has proven that it is a research model for various molecular, cellular, and toxicological experiments. The model system facilitates to showcase of the long-term and short-term effects of extensive toxicological agents with less intricacy. Zinc, cadmium, selenium, mercury, copper, nickel, iron, cobalt, lead, chromium, aluminium, organics such as phenols, amline, cyclohexane, and their derivatives falls under the category of compounds whose toxicological studies have been concluded using *Danio rerio* [171]. With the scientific discoveries demonstrating genetic, cellular, anatomical, and physiological resemblances between zebrafish and

mammals, the zebrafish model has gained recognition as a suitable model for toxicological studies [172]. Zebrafish being a small tropical aquarium fish ranging from 4–5 cm, commonly found in rice fields, having navy and horizontal stripes running through the length of the body from caudal fin to gills, has broader dimensional advantages [173] [174].

4.1. Advantages

The unique characteristics and remarkable attributes of zebrafish, such as their high fluidity rate, rapid embryonic growth, and transparency of the embryo, contribute to their exceptional value. These features enable researchers to observe the various stages of the cell cycle and development externally. A single pair at optimal temperature, pH, conductivity, and food supplement can assure 300 to 600 fertilized eggs in single breeding [173–176]. The year-round sexual activity of zebrafish enhances their suitability as a research model due to their consistent reproductive capability. Phenotypic evidence, such as external fertilization and development, can be readily observed under an ordinary light microscope in zebrafish, unlike in mice. This unique characteristic of zebrafish reduces ethical concerns associated with embryo experimentation and allows researchers to conduct experiments continuously.

Despite the morphological dissimilarities with exceptions like lungs, placenta, and mammary gland, all essential organs of humans are found in zebrafish [174] as they possess the identical cell types, genes readout the same identity and functions in both. Seventy-one percent of all human genes matching with zebrafish have been identified and the homology of human disease genes accounts for 80% with zebrafish. Brain neurochemistry is highly conserved among vertebrates, Zebrafish possess all major neuromediator systems including receptors, transporters, and enzymes [174,177].

The feature of having external fertilisation in zebrafish makes easier for examining gene function and builds a bridge for genetic manipulations which aids in turning off or down-regulating a specific gene. For example, embryos mutated with the MY018B gene set out to be a model for studying human myopathy, and pKd2 mutant embryos were employed for polycystic kidney disease. External fertilization helps immensely to produce transgenic zebrafishes using a T transpoon system which initially came from medaka fish, the process includes the introduction of two minimal as regulatory sequences from the T element at 5'end, 3'end of a minimal promoter followed by a fluorescent protein. Tol2 containing Viton has the potential of oblinging DNA, inserting up to 11Kb. The newly made functional gene along with the mRNA coding gene is pushed into a one-cell stage of the embryo. They are further raised as transgenic fishes which serve as an important basis for studying hematopoietic stem cells and prog rator cells. CRISPR/Cas9 technology creates mutants of desired varieties [178].

4.2. Role of zebrafish embryos

Contemplating the phases of embryonic development of the Zebrafish is a tool that offers precision in anatomical and hereditary methodologies. The fertilization occurs externally and involves the fusion of sperm from the male to an egg of the female. Gametic cells start to develop, and incipient organisms fundamentally are considered in this phase until they hatch or escape the chronic. Cleavage is a phase in which rapid miotic divisions happen. The zygote is separated into numerous cells and toward the finish of the cleavage arrangement, the blastula has shaped. In the gastrula stage, the pace of mitotic division turns out to be delayed in creating the various organs. Somites are created during the segmentation phase [179,180]. Pharyngula stages are portrayed by spontaneous movements, separation from the yolk, early pigmentation, retina pigmentation, cell degeneration of the last part, dissemination; single aortic each pair, early motility, and heart beating.

The hatching period is extended from 48 h to 72 h post-fertilization (hpf) [181]. After hatching, it's characterized as a Larva. The other features include regularity of heartbeat, yolk expansion, dorsal and ventral pigmentation, segmental veins detectable; thickened sacculus with two chambers visible, and foregut development. In the Juvenile phase, most grown-up highlights have been shaped without sexual productivity. Adult stages begin at multi-day post-fertilization (dpf). The utilization of lab creatures, for example, rats, mice, and rabbits have been considered the best quality level in herbal toxicity assessment. However, in recent decades, the ethical considerations surrounding the use of higher vertebrates for toxicity testing have become increasingly contentious. In this context, conceivable elective models involving lower vertebrates, for example, zebrafish are introduced. The embryotoxicity model of zebrafish is at the forefront of toxicology assessment due to the transparency of the embryos, cost-effectiveness, short reproductive cycle, high fertility, and genetic accessibility to the population. In recent times, the application of the zebrafish embryo model has been expanded to include herbal toxicology.

The small size of the embryos makes them highly useful for the large-scale screening of new drugs. The newly discovered drugs can be diluted into varying concentrations in 24, and 96-well plate systems. The embryos can be placed into the wells of ELISA trays or plates. The embryos assimilate test samples from the surrounding water medium through their plasma membrane, and the toxicity results are generated within a specified timeframe [174,177]. Aripieazole, clorapine, olanzapine, risperidone, and ziprasidone are the first-generation antipsychotics that were evaluated using *Danio rerio* embryos. Parameters such as tail and body malformations, edema, and heartbeat rate are carefully evaluated, with particular emphasis on heartbeat rate as it serves as a fundamental basis for risk assessment assays [182].

Zebrafish embryo model has been utilised for the evaluation of herbal toxicity. An experiment was conducted which highlighted the toxic effect of Zinc oxide nanoparticles on the cell cycle of zebrafish. cDNA microassay was performed to evaluate the genotoxicity of Zinc oxide. The nano form of zinc oxide primarily impacts the cell cycle pathway in zebrafish. The suppression of cyc/cdk complex expression results in disruption of the cell cycle progression [183,184] (Fig. 7).

4.3. Toxicological endpoints in Zebrafish

The *Danio rerio* vertebrate model has emerged as promising model to provide valuable insights into the pathways of toxicity development, thereby enhancing our understanding of human health risk assessment [190]. In toxicological testing, the behavioural aspect of zebrafish is regarded as a significant parameter, as deviations from their normal phenotypes indicate abnormalities in their typical physiology. Both the larval and adult stages of zebrafish actively engage in behavioural responses. When drawing conclusions from a toxicity assay, various toxicological endpoints such as angular development, tail malformations, body heartbeat rate, pigmentation, and more are taken into account. Additionally, two pioneering methods in high-throughput analysis, namely photo motor response (PMR) and virtual motor responses (VMR), are considered. When zebrafish embryos are exposed to a light source between approximately 24 to 30 hpf, these methods can induce a reflex response via the hindbrain pathway and result in heightened activity levels. Upon exposure to chemicals, the larva shows deflection in duration, and the velocity of movement falls under (VMR) [191]. Toxicological assays typically span a duration of five days during which toxicological endpoints are consistently monitored. The vast potential of the Zebrafish model is set to be harnessed on a significant scale for the advancement of scientific research and the improvement of society as a whole (Fig. 8).

4.4. Zebrafish genome

The *Danio rerio* as a potential genetical model came into a scenario in the year 1980. Systemic applications of genetics supported a large

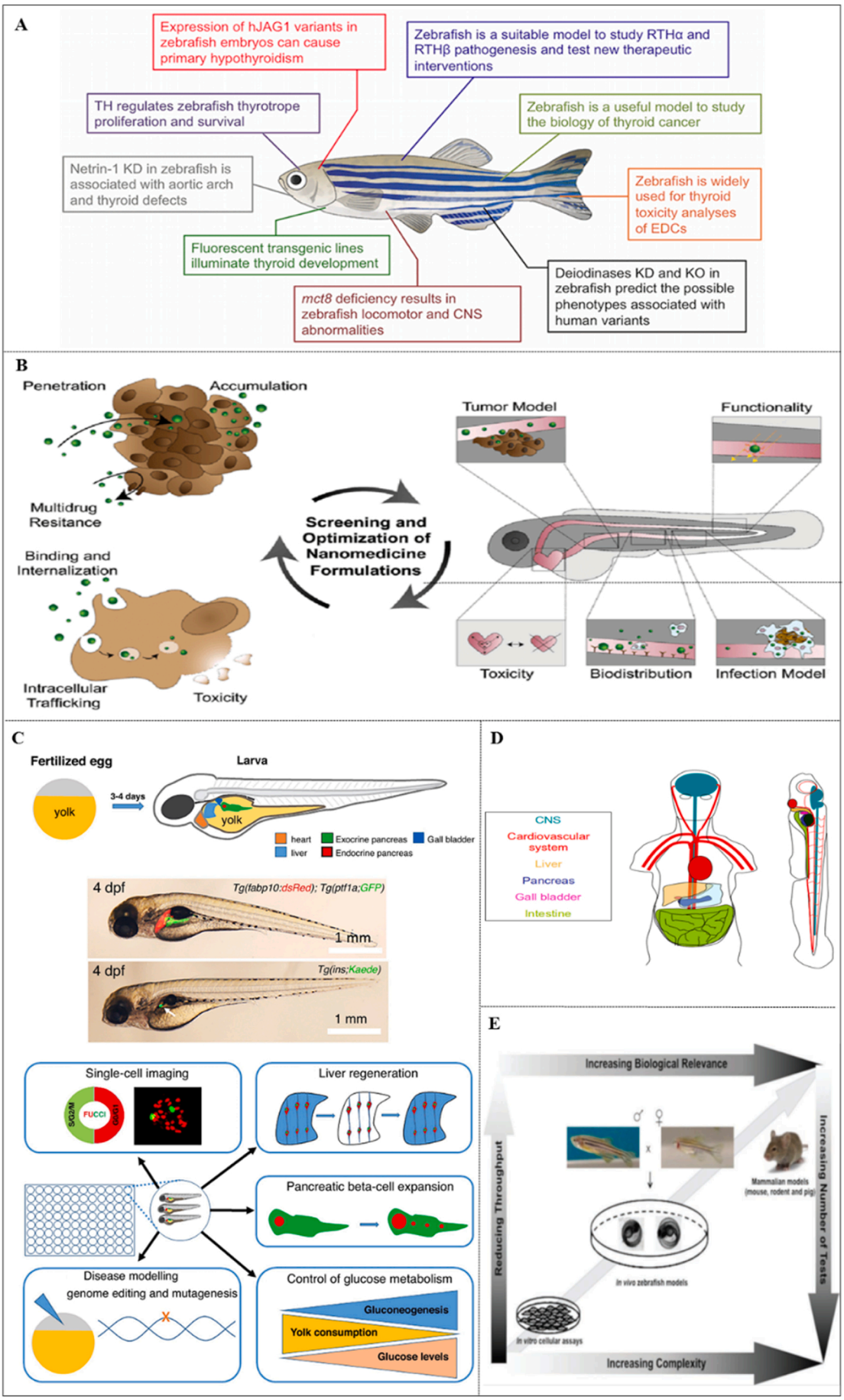

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Fig. 7. (A) Schematic illustration of the most recent findings concerning thyroid development and thyroid hormone (TH) action that have been obtained using zebrafish as a model system. (CNS), central nervous system; (EDC), endocrine disruptor chemical; (KD), knockdown; (KO), knockout; mct8, monocarboxylate transporter 8;(RTH), resistance to thyroid hormone [185]. (B) Complementary application of in vitro and zebrafish model experimental set-ups for nanomedicine formulation design and optimization. The complementary application of classical 2-D (bottom left) and sophisticated 3-D (top left) in vitro systems and the zebrafish model (middle right) offers the possibility to assess nanomedicine interactions with biological environments under complex biological conditions. The availability of an optimized preclinical, in vivo screening platform [186]. (C) An overview of the zebrafish model: Breading a single pair of adult zebrafish can give rise to large clutches of fertilized eggs that develop externally into larvae with functional organs, including liver and pancreas [187]. (D) Conservation of organ systems between zebrafish and humans [188]. (E) High fecundity, embryo transparency, highly conserved cellular and metabolic activities, etc., zebrafish offers higher biological relevance and complexities compared to in vitro cellular assays, while maintaining high throughput and high volume data generation capabilities [189].

discovery of phenotypic traits of mutants [90]. *Danio rerio* has 25 chromosomes and the genome comprises ~ 1.5 × 10 billion base pairs [195]. The studies have suggested that there are more than 26000 protein-coding genes in Zebrafish. 71.4% of the human gene's at least one Zebrafish orthologue. One-to-one human genes orthologue with Zebrafish accounts for 47%. The second highest orthology of (one human gene-to-many-Zebrafish) with a mean of 2.28 Zebrafish genes for each human gene offers thought to telecast-specific genome duplication [90]. Repetition in the genome is reportedly found highest in Zebrafish than in other vertebrates. A scientific team identified 154 pseudogenes in the Zebrafish genome, a fraction of 13000 pseudogenes found in the human genome (Fig. 9).

4.5. Use of Zebrafish models to study human diseases

With rapid human development, deadly diseases are evolving and such human disease studies can be accomplished by deploying the Zebrafish model. *Danio rerio* has 2 eyes, a mouth, a brain, a spinal cord, an intestine, a pancreas, a liver, bile ducts, a kidney, an oesophagus, a heart, ear, nose, muscle, blood, bone, cartilage, and teeth. The cellular and genetic composition of the organs mentioned above exhibit a high degree of conservation between humans and zebrafish. As a result, alterations in body shape and functions observed in *Danio rerio* due to diseases can be replicated in humans as well [198]. Advancements in tools and technology like CRISPR/Cas 9, mass spectrometry-based polar metabolomics and lipidomics, and in vivo imaging fluorescent dyes have made it easy to explore and study human metabolic diseases like obesity, type 2 diabetes mellitus, non-alcoholic steatohepatitis, and atherosclerosis in Zebrafish [199]. An experiment has proposed that subjecting Zebrafish embryos to ethanol is now widely recognized as a standard approach for studying fetal alcohol spectrum disorders resulting from maternal alcohol consumption during pregnancy [200]. The integration of the Zebrafish model with CRISPR-induced null mutation in atp6V1h has been devised to investigate the effects of ATP6V1H loss of function on bone homeostasis [201]. Human heart disorders, muscular dystrophy, nephronopthises, central nervous system disorders, and ocular disorders are well-studied in the zebrafish model. Forward genetics have been employed to point out mutants with heart defects like human dilated cardiomyopathies (DCMs) characterized by enlargement of the ventricle and artery and reduced myocardial contractility. Muscular dystrophy was concluded as a lethal genetic disorder after a mutating dystropin gene (called dmd and sapje) in zebrafish. Effects of drugs such as rapamycin and roscovitine ameliorate on kidneys have been reported using morphant embryos. Zebrafish mutants with visual impairments provide insights into the involvement of signaling pathways, such as hedgehog (shh), nodal, and retinoic acid, in both eye disease and development, which is relevant to humans. *Danio rerio* model is gaining recognition as a versatile resource for investigating disease biology and facilitating drug discovery [202] (Fig. 10).

5. Nanomaterial-induced toxicity assessment in zebrafish

The emerging field of nanotechnology is expanding expeditiously due to its wide range of applications. Nanoparticles (NPs) /Nanomaterials (NMs) are one of the most valuable components of this field due to their unique properties and significant applications including targeted drug delivery for diagnostic purposes **(**Table 4**)**. The size range of inorganic NMs (e.g. metal and metal oxides like Au, Ag, TiO_2, etc.) is between 10 nm to 100 nm whereas larger particles up to 1000 nm to 2000 nm in aqueous suspensions have been observed, But unfortunately, the small size and increased surface to mass ratio reveal heightened cytotoxic properties [204] to be used for diagnostic or biomedical purposes. For the cellular level toxicity assessment, the simple organisms and cell lines are useful whereas, for the detection of complex physiological interactions, the higher vertebrates are required [205]. In such a scenario, the exploitation of the zebrafish model for screening toxicants has captivated attention. Toxicological experiments with zebrafish aid to detect not only harmful heavy metal pollutants present in the environment but can also be used for monitoring organic pollutants such as microplastics, pesticides, and antibiotics [15]. The Fish Embryo Toxicity (FET) assessment is integrated with the performing toxicity tests concerning the FDA (Food and Drug Administration) and ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) for pharmaceutical products. Additionally, regarding the chemical substances, FET can also be performed for EPA (the United States Environmental Protection Agency) and OECD (Organization for Economic Co-operation and Development) [206]. Following the OECD guidelines, various toxicity assessments have been performed on zebrafish. In most of the assessments related to NMs, the authors have reported developmental malformations like hatching delay and malformation, and deformity in the body part, causing mortality.

Nanomaterials may also possess the noteworthy property of biocompatibility which serves the welfare of society thus making it essential to study the potential toxicity caused by NMs, especially the biocompatibility assessment of much used Ag NPs (silver nanoparticles); they are deployed in diverse fields, including health care, medical, food, consumer, and industrial purposes [220]. Verma et al. conducted a study on the toxic impact of Ag NPs synthesized through a novel one-step rapid method, employing two Gram-positive bacterial strains (*B. thuringiensis* and *S. aureus*) and two Gram-negative bacterial strains (*E. coli* and *Salmonella typhimurium*) [218]. The biogenic Ag NPs were found to be toxic since the pericardial edema with swelled gastrointestinal lumen along with severe effects was observed in 72 hpf embryos. In another study, Ag NPs were exposed to adult zebrafish in which gene expression assessment was performed in liver tissues [221] which resulted in the upregulation of *HSP70* and *NFKB* genes whereas downregulation of *MTF-1*, *TLR4*, *IL1B*, *CEBP*, *TRF*, and *TLR22* genes. The outcomes concluded that oxidative stress and immuno-toxicity could be increased by Ag NPs. In an investigation, the embryonic zebrafish were treated with TiO_2 NPs and bulk TiO_2 [219], wherein relative to bulk TiO_2, numerous effects of treating embryos with TiO_2 NPs revealed deformation in chorion and yolk sac in 48 hpf whereas, in 96 hpf deformations in the heart development, tail, and notochord were observed. Gold nanoparticles (Au NPs) also have a varied range of applications including cellular labeling, and drug delivery, and most importantly it is also used for imaging and diagnostics of diseases like diabetes, Alzheimer's, and cancer [222]. Numerous investigations of AuNPs toxicity screening have been performed on the zebrafish model and the results showed less toxicity when embryonic or adult zebrafish were exposed to AuNPs [223,224]. Whereas in another experiment, S. Kumari et al. studied the exposure of green synthesized Au NPs (LC50 estimated at 116 μg/mL) in embryonic

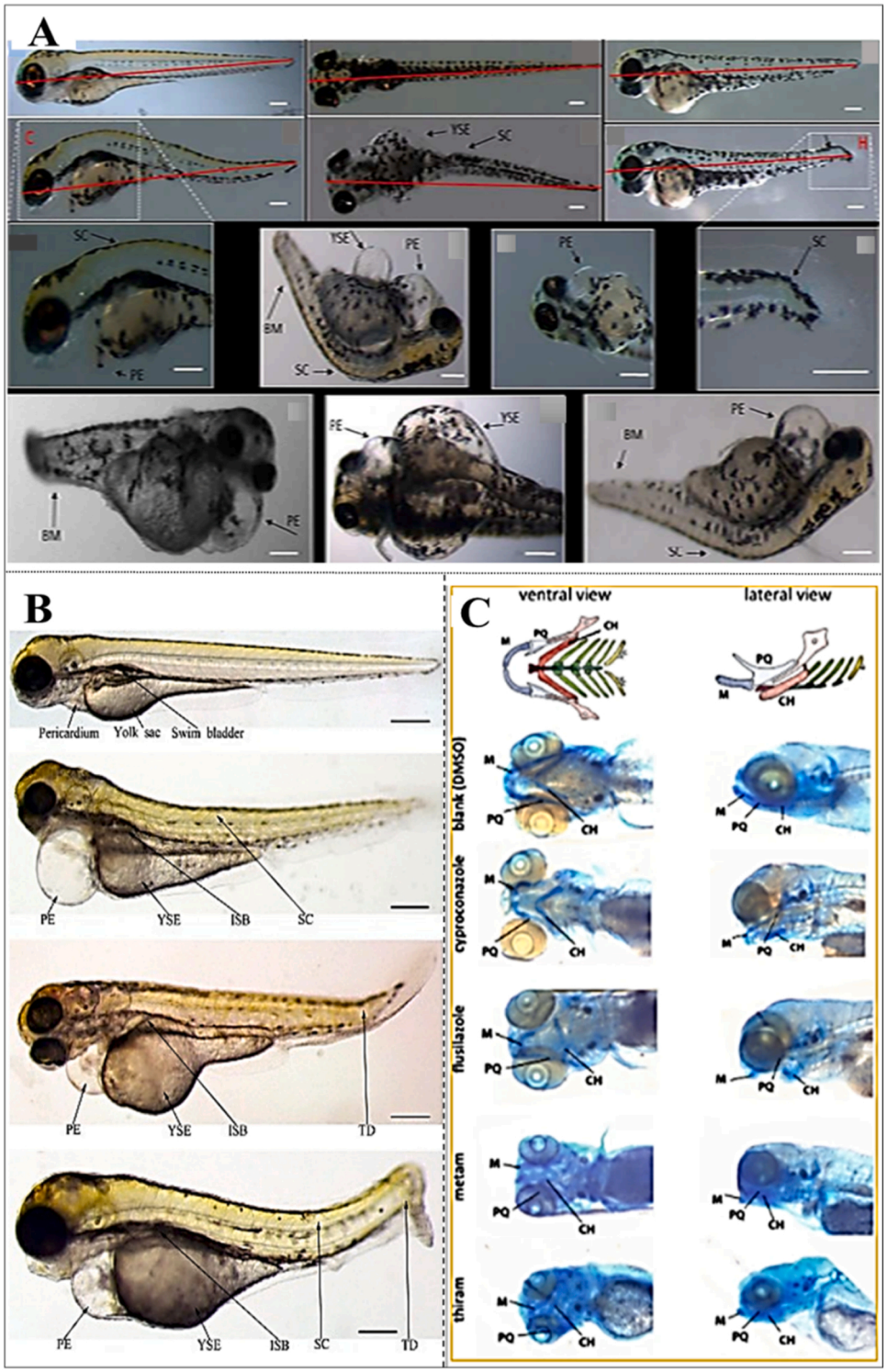

Fig. 8. (A) Microscopic images of embryos. Control embryos (96 hpf), (96 hpf) and (72 hpf); 10 mg/L glyphosate treatment groups (72 hpf), (96 hpf), (72 hpf); 100 mg/L glyphosate treatment groups (72 hpf), K, M and N (96 hpf). YSE: yolk sac edema; PE: pericardial edema; SC: spinal curvature; BM: body malformations (including tail malformation, short tail and head malformation). Scale bar: 200 μm [192]. (B) BBP induces developmental toxicity in zebrafish embryos. BBP has adverse effects on the heart development in zebrafish embryos. BBP alters the expression of Nkx2.5 and Tbx5 in zebrafish embryos [193]. (C) Head cartilage structures of alcian blue-stained zebrafish embryos in ventral and lateral views after exposure to DMSO only (solvent control), cyproconazole (60 μM), flusilazole (10 μM), metam (10 μM) or thiram (0.3 μM), at the highest non-lethal concentrations [194].

zebrafish [217]; AuNPs were synthesized using aqueous leaf extract of *Andrographis paniculata* (*A. paniculata*) and hence termed as green synthesized AuNPs. The aggregation of AuNP at the chorion created unwanted hypoxic conditions leading to metabolic disturbances. The deformities like the malfunctioned eye, bending tail, and edema were observed from the respective results of the experiment. In another study, the pathways involved in inflammatory and other immune responses were disrupted by AuNPs [225]. Zebrafish embryos when exposed to CuO NPs synthesized by the green method via the flowers of *Calotropis gigantea*, lead to a swelled yolk sac, notochord bending, and pericardial

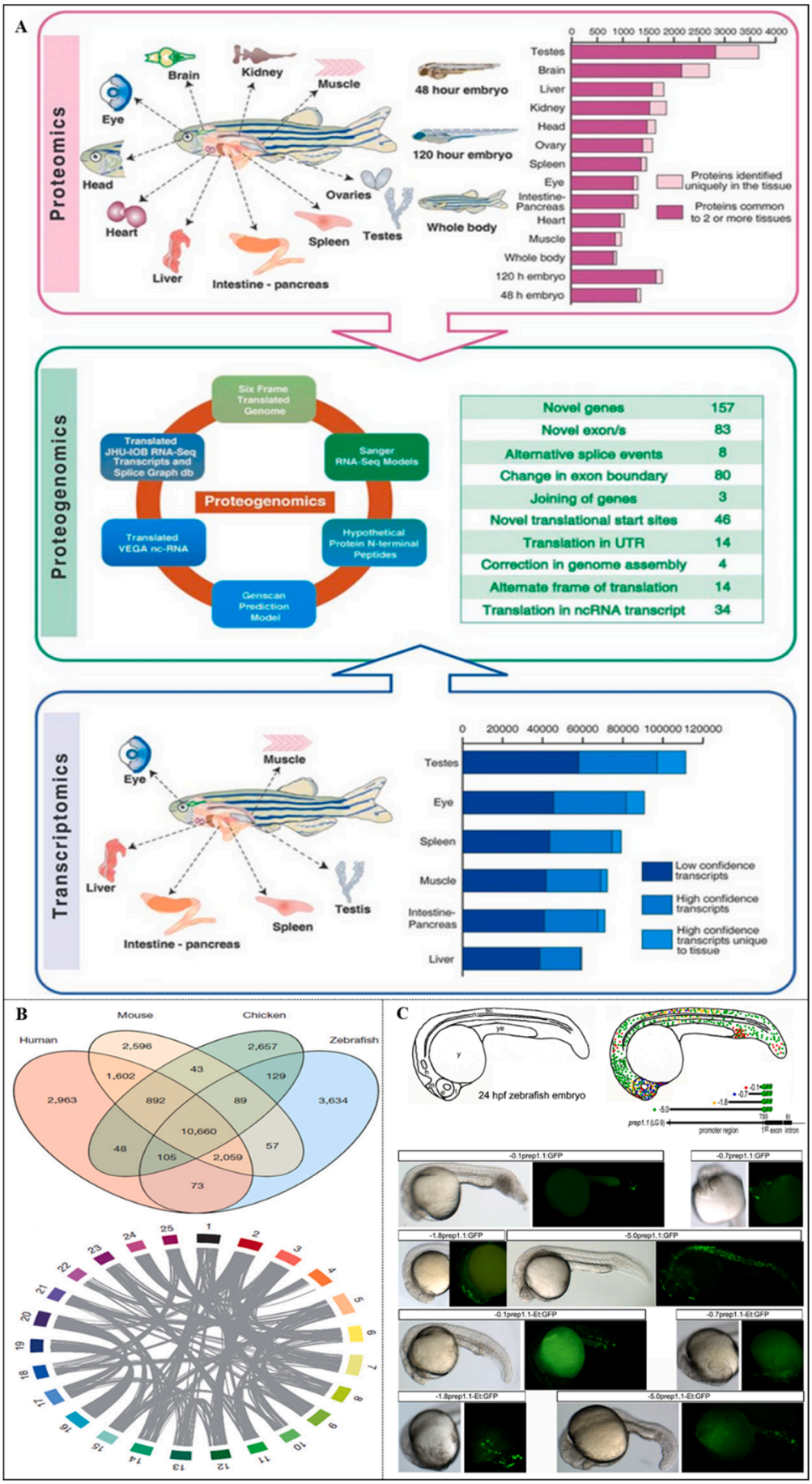

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Fig. 9. (A) Integration of transcriptomic and proteomic data for improving genome annotation. Adult zebrafish organs and developmental stages used in proteomic analysis and total proteins and sample-specific proteins identified in the study. Seven alternative databases used in proteogenomic analysis and summary of proteogenomics findings. Adult zebrafish organs used in transcriptomic analysis, total transcripts, high-confidence transcripts, and sample-specific transcripts identified in each organ [196]. (B) Orthologue genes shared between the zebrafish, human, mouse and chicken genomes, using orthology relationships from Ensembl Compara 63. The ohnology relationships between zebrafish chromosomes. Chromosomes are represented as coloured blocks. The position of ohnologous genes between chromosomes are linked in grey (for clarity, links between chromosomes that share less than 20 ohnologues have been omitted). The image was produced using Circos30 [90]. (C) Transient expression of zebrafish prep1.1:GFP promoter constructs in 24 hpf zebrafish embryos [197].

edema [226]. Moreover, it was found that CuO NPs affected the Sod1 and p53 enzyme functionality due to which such deformities were observed. Similar effects were noted when embryonic zebrafish were exposed to green synthesized MgO NPs [216]. Verma et al. have helped to gain information regarding the cytotoxicity assessments and the biocompatibility of several NMs synthesized via the deployment of various plant components [227]. Apart from features like biocompatibility and high antibacterial efficiency, the green synthesized NPs have also exhibited toxic properties. Various factors like the synthesis process of NM, the concentration of NM, and other environmental conditions influence the NM-based toxicity assessment where the zebrafish model has successfully assisted to demonstrate such toxic properties of NMs (Fig. 11).

6. Transcriptional profiling and high-throughput screening (HTS) for toxicity studies

The zebrafish model is most frequently used for carrying out transcriptional profiling and high-throughput screening (HTS) for toxicity studies. In an investigation by Haggard et al. [228], zebrafish embryos were exposed to 25 estrogen-, androgen-, or thyroid-active chemicals at several concentrations, 80% of the animals were found to have suffered unfavorable abnormalities or died. Also, the thyroid hormone receptor agonists were found to have a distinct transcriptional and phenotypic signature when compared to all other treatments. Additionally, the study proposed the possibility of employing a tiered in vivo HTS based approach on this distinctive signature. This approach holds promise for discovering molecules that interact with the thyroid hormone receptor and can assist in the identification of such substances. The Vertebrae *Danio rerio* model, has gained substantial recognition as the leading choice for high-throughput toxicity testing in developing novel drugs and pharmaceuticals [125].

The Zebrafish model can provide valuable insights on the establishment of toxicity pathways [190]. After exposure to chemicals, the duration of the larva's movement deflects, and the velocity of the larva's movement falls into the (VMR) category [191]. Several procedures have been developed to enable high-throughput measurement of heart rate, contractility, and blood flow in zebrafish. Additionally, various secondary assays, including optical voltage mapping, Ca2 + imaging, specific transgenic reporters for subcellular Ca2 + compartments, discrete signaling reporters, and even organelle function, have been employed to enhance the analysis of zebrafish physiology [229]. The complete immersion of zebrafish embryos during in-water dosing can lead to exposure to a wide array of substances that are uncommon in mammals. The administration routes shift from fish to mammalian models, and as a result, the data gathered in zebrafish cannot be transferred to other animals, including humans, in a realistic manner. In the case of insoluble or weakly soluble compounds, the in-water dosing method is inadequate for effectively exposing the embryos to these substances. To ensure proper exposure, such compounds need to be directly injected into the fish. When screening zebrafish embryos at high throughput, this must be accomplished. This model organism should not be regularly used to study genotoxicity, biodistribution, pharmacokinetics, pharmacodynamics, or transcriptional profiling because of these limitations.

7. Drug discovery toxicology: value for replacement, reduction, and refinement

Widespread skepticism about the possibility of replacement, particularly in the activity and toxicity studies, can be attributed to the "three Rs" [230]. Fundamental pharmacology and toxicology made tremendous strides during the 1980 s and early 1990 s, thanks to advancements in tissue culture methods during that time. However, except for one innovative new statistical technique for lowering the number of animals required in vaccine testing, there has been little success in the decrease of animal testing. The use of the Three Rs to work with primates is a future goal that ought to be pursued. New applications for experimental animals continue to emerge, but recent triumphs have given us reason to be optimistic about the future of humane experimental procedure as it continues to advance. The Zebrafish model system follows the **3 Rs** of animal model research studies [231,232];.

- **Replacement:** Zebrafish assays employing larval zebrafish have the potential to serve as alternatives to certain animal toxicity studies. However, prior to adoption, it is crucial to establish the relevance of the larval zebrafish model for the specific system (target, gene, pathway, mechanism, tissue, organ, etc.) through rigorous validation studies.

To make significant progress in replacement, scientists who are intimately familiar with their particular field and who can analyze the viability of certain replacement solutions, are typically required to take the lead. The use of replacement technology frequently enables scientists to do investigations that would be impossible to conduct with traditional animal models. One example is the innovative technology known as the human 'gut-on-a-chip,' which replicates the natural conditions of the human intestines in a small-scale, programmable, in vitro platform. Another major advancement is the utilization of human microfluidics technology. As a result, it has become feasible to co-culture intestinal cells with live bacteria derived from both the normal and pathological microbiome over an extended duration. This approach also offers a valuable in vitro model for studying enteric virus infection and exploring the mechanisms underlying enterovirus pathogenesis. These investigations would otherwise be unattainable using animal models or static cell culture techniques. Although increasing the number of animals used to verify scientific validity may be necessary to build trust in a replacement approach, the increased use can be justified by the eventual decline in animal suffering. For example, substituting the traditional mouse bioassay for marine biotoxins in shellfish necessitated scientists identifying the relevant toxins using appropriate analytical technologies such as high-performance liquid chromatography-mass spectrometry (HPLC-MS). Following that, rigorous validation studies were conducted to verify that human safety would not be jeopardized because of the use of non-animal technologies. As a result, a series of more accurate tests have been developed, which fully replace a mouse model that resulted in death and paralysis.

- **Reduction:** Zebrafish larvae, being a reliable indicator of toxicity, can be employed as an initial screening tool to identify potentially harmful drug candidates. By using zebrafish larvae, safer drug candidates can be screened before moving on to testing in mammalian models. This approach would ultimately lead to a reduction in the number of animals required for testing purposes. For many years, it has been recognized that lowering the number of animals utilized to the bare minimum may not be the most ethical or welfare-conscious course of action. Primarily, employing a small number of animals can yield data that are statistically arduous or impractical to interpret effectively.

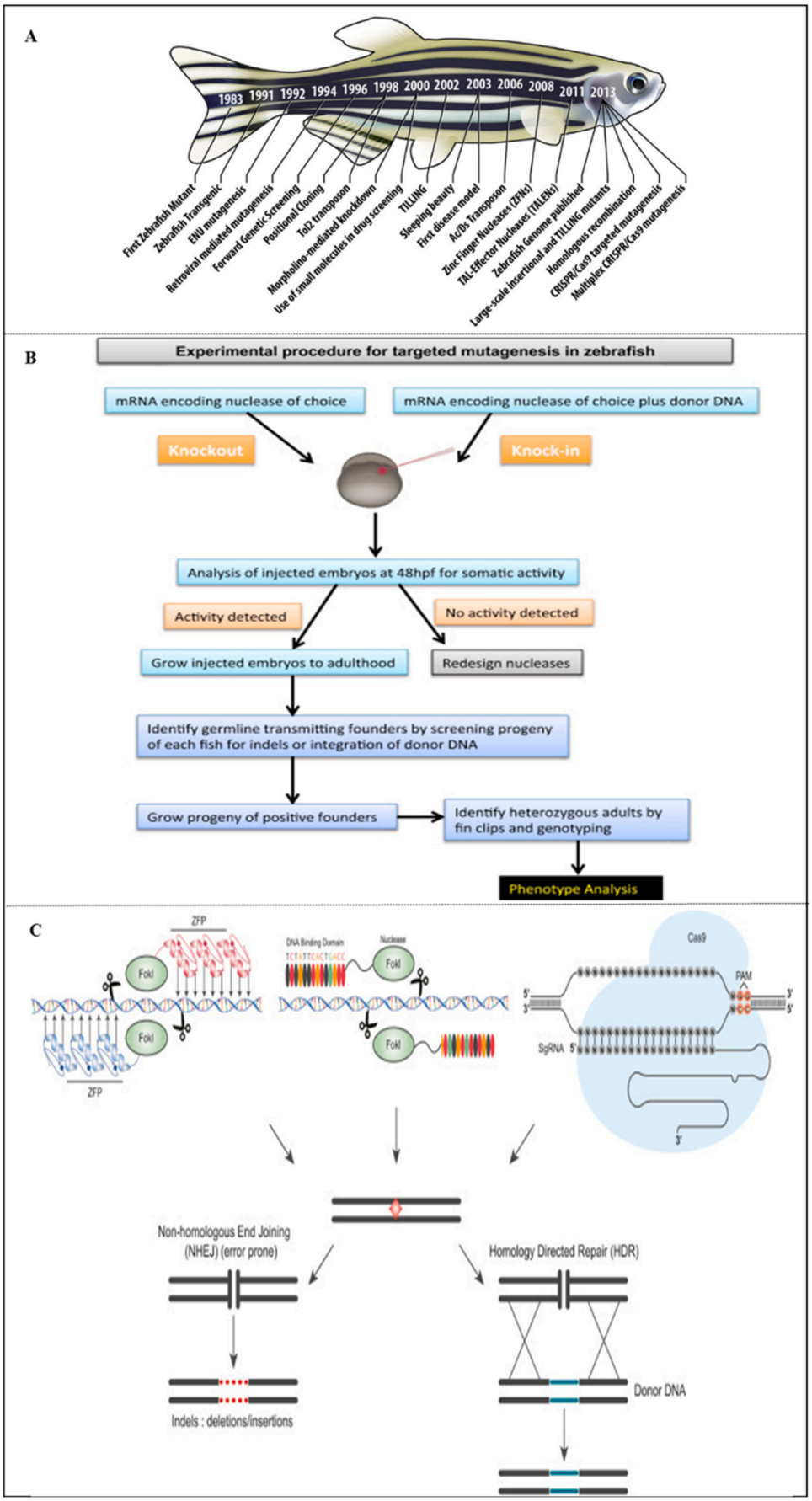

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Fig. 10. (A) Timeline of important technological developments in zebrafish research [203]. (B) Overview of the experimental procedure for targeted mutagenesis in Zebrafish [203]. (C) Schematics of Genome Editing by ZFNs, TALENs, and CRISPR/Cas9. All nucleases essentially generate a double-stranded break (DSB) at the target site. These DSB can either be repaired by error-prone non-homologous end joining (NHEJ) which often leaves indels, or if a donor template is supplied, then the DSB can be repaired (hopefully) perfectly by homology directed repair (HDR) [203].

Consequently, it becomes essential to strike a balance between minimizing the overall number of participants and obtaining valid data, all while ensuring that individuals are not subjected to unnecessary harm. Due to this, the modern perspective on 'Reduction' places greater emphasis on experimental design and strives to maximize the reproducibility of outcomes whenever feasible. A variety of causes have been advanced, including the use of insufficient cell lines and mice models. The low quality of experimental design and published results, on the other hand, has been a significant factor. The studies in which the findings could be replicated stated how close attention was paid to controls, reagents, and investigator bias, and included the entire data set in the analysis, among other considerations. This matched observation made by Ioannidis and Prinz et al. earlier in the year. Furthermore, 90% of the more than 1500 scientists who took part in the survey judged a lack of reproducibility to be a crisis of either substantial or minor dimensions. This represents an ethical dilemma for the scientific community around the world, particularly in cases where animals have been utilized in the research.

• **Refinement:** In addition to our ethical responsibility to maximize well-being, the scientific case for 'Refinement' is compelling. With the advent of modern non-invasive imaging techniques, it is now possible to monitor subtle changes over time without resorting to the sacrifice of animals to obtain samples. Although these treatments yield positive results, they often entail the repeated sedation or anesthesia of animals, highlighting the importance of acknowledging the potential discomfort it may impose. Fortunately, training animals to voluntarily undergo non-invasive procedures while remaining conscious can offer a solution to mitigate this problem.

In summary, embracing the principles of the 3Rs in the research holds the potential to deliver not only ethical advantages related to the reduction of animal usage and enhanced animal welfare but also the opportunity to yield scientific benefits in various aspects. When compared to animal testing, many replacement technologies provide more consistency and precision while also providing faster results besides being often less expensive. Aside from that, numerous studies of this nature are impractical to conduct using animal models, either due to the limitations of low-throughput animal models or the necessity for tissues that accurately represent the target human species. More importantly, adhering to stringent criteria in experimental design to ensure repeatability, alongside the principle of 'Reduction', leads to improved scientific outcomes. In addition, by following the principles of Refinement to the letter, stress as a scientific variable is kept to a minimum. Thus, the 3Rs create a potential for high standards of animal welfare to coexist with better science, faster science, and more cost-effective science, all of which contribute to improved animal welfare. Also, implementing 'Replacement' techniques provides the added advantage of reducing the burden of regulatory scrutiny necessary to ensure that the use of animals is ethically justified and scientifically warranted. When animal use is proposed, it's critical to eliminate needless bureaucracy to offer timely, fair, and defensible oversight judgments that are based on solid scientific evidence.

8. Challenges of zebrafish as an animal model

Zebrafish has been recognized as an unique model due to the uniqueness in properties and its significant features, like rapid development, and short generation time, among other attributes, however, this model has several drawbacks compared to the mammalian models [233]. The compact size of zebrafish, which offers several advantages as a model organism, also presents certain challenges, such as the embedding and sectioning of embryos, and the selection of appropriate recording equipment for testing and evaluation purposes [234]. Hence the size of the larvae is a great constraint while working with it. Due to this limitation, non-clinical toxicological studies are relying on lesser efficacy[235]. Also, toxic exposures, dosage analysis, distribution, absorption of chemicals, etc. could not be extensively studied as the small size presents a challenge. The measurement methods for plasma levels and excretion data in embryos have made limited progress due to the above limitations, and they are still in their nascent stages [236,237].

Restrictions and issues remain regarding the translatability of toxic potencies and affected tissues. Usually, for dosing the zebrafish embryos, the in-water technique is used [17]. Firstly, since the embryos are immersed completely in the given solution, the in-water dosing of zebrafish may cause them unique exposures compared to mammalian routes. Secondly, insoluble chemicals or the ones with poor solubility fail to enter the embryos through in-water dosing but are needed to be injected directly into the fish to expose them sufficiently to those chemicals. This needs to be done for high throughput screening of the zebrafish embryos. The rapid growth and development which once appeared to be a boon for using zebrafish as a model organism have now several challenges associated with their developmental studies. This rapid pace of development needs very minute observations throughout the entire post-fertilization period to record the data because even a few hours can make a large difference or change in pattern in a developmental stage depending on the sensitivity of the embryos towards the chemical treatment being given [234]. Almost all the major organ systems, including larval swimming, vision as well as neuronal pathways, the central nervous system, the spinal cord, etc. are developed within a very short period.

Further, in terms of mutation, the zebrafish failed to facilitate the advantages provided by mouse models. In mouse models, the gene of interest can be readily mutated and introduced by homologous recombination[238]. But the transposon system has enabled easy insertion of transgenic sequences into the zebrafish genomes which further simplified the manipulation of fluorescent proteins, Cre- recombinases, cell-death proteins, etc. to utilize them and bring out new approaches [239–241]. The search for simpler techniques has continued and with time newer methods have evolved wherein genes of interest alterations were possible via some antisense RNA mechanisms. However, not all genomes could be easily altered. There were still some issues unresolved since numerous phenotypes produced by morpholinos did not respond to the gene-editing techniques due to pronounced off-target effects. Thus, further studies have been suggested for more careful replication of the morpholino-based data [242]. Comparative phylogenetic data on anatomy and physiology differences indicate that, in general, the zebrafish model produces less clinically relevant data concerning toxicities compared to mammalian models. Lastly, after considering a lot of facts regarding gene similarity, cellular mechanism, etc. the toxicological studies involving zebrafish, also need to consider their regeneration potential. Zebrafish can regenerate their fin, brain, retina, spinal cord, heart, kidney, pancreas, liver and more which creates a huge impact on the translational toxicity endpoints [243–245].

Table 4
Biocompatibility and toxic effect of several nanoparticles (NPs) using the zebrafish model system.

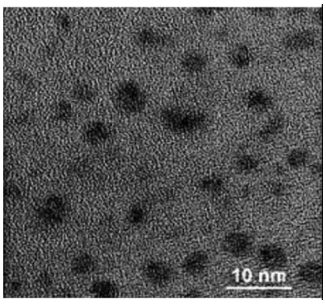
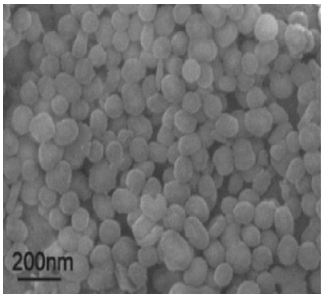
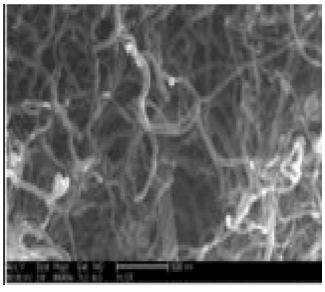
Sl. No.	Nanoparticle (NP)	Bio-compatibility	SEM/TEM/FESEM Images of NPs	Toxicity observed in Zebrafish	Reference
1	Carbon Dots	Shows higher biocompatibility than other nanoparticles		At higher concentrations, delayed development, inhibition of pigmentation, pericardial edema, delayed hatching observed.	[205, 207]
2	Graphene Quantum Dots (GQDs)	High biocompatibility		GQDs were found to accumulate in the digestive system, while the blood, muscle. Also, affects growth at lower concentrations.	[205, 208]
3	Pristine CNTs (carbon nanotubes)	High biocompatibility		Showed little effect on embryo viability and development, even at high concentrations.	[205, 209]

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Table 4 (*continued*)

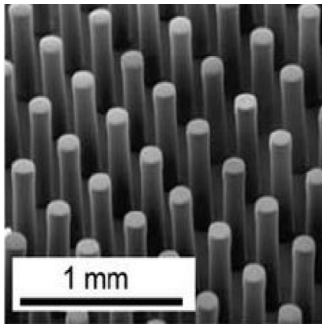
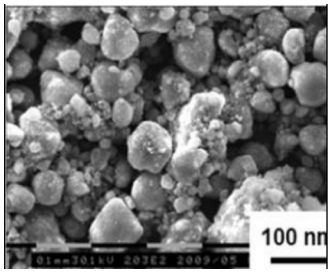
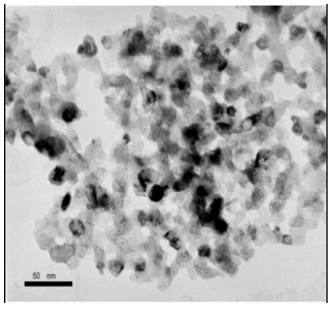
Sl. No.	Nanoparticle (NP)	Bio-compatibility	SEM/TEM/FESEM Images of NPs	Toxicity observed in Zebrafish	Reference
4	Single-Walled CNTs (carbon nanotubes)	High biocompatibility		Increased mortality, delayed hatching, and decreased total larval length only at the highest concentration tested (1 ppm).	[205, 210]
5	Aluminum nano-particles (AlNPs) and Al2O3NPs	Highly biocompatible		Decreased Na+ , K+ -ATPase activity in gills and changes in expression of two genes.	[15,211]
6	Magnesium Oxide (MgO)	Moderately biocompatible		Induced cellular apoptosis and intracellular reactive oxygen species. Hatching rate and survival of embryos decreased with higher doses.	[15,212]

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Table 4 (*continued*)

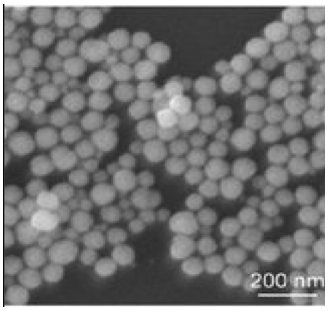
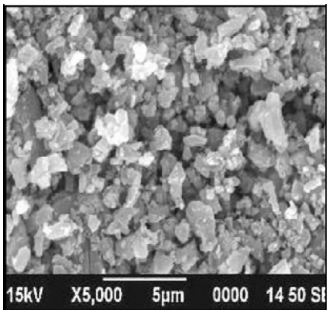
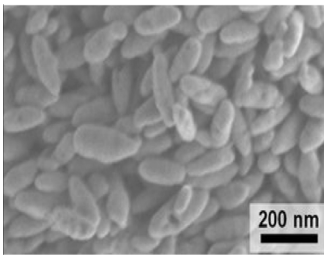
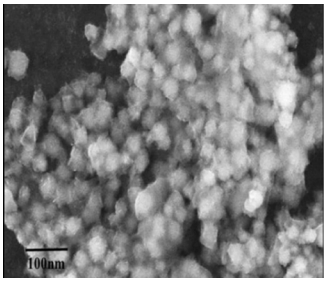
Sl. No.	Nanoparticle (NP)	Bio-compatibility	SEM/TEM/FESEM Images of NPs	Toxicity observed in Zebrafish	Reference
7	Platinum (Pt)	Unknown		Neurotoxicity in the zebrafish model of Parkinson's disease via its 2- functions as mitochondrial complex I and antioxidant activity (SOD and catalase mimic activities).	[15,213]
8	Nickel (Ni) and Nickel Oxide	Moderately biocompatible.		Showed that, the acute toxicity of NiONPs was low but chronic exposure of NiONPs could lead to the accumulation and increase in toxicity in zebrafish tissue.	[15,214]
9	Iron Oxide (Fe_2O_3)	Highly biocompatible.		Highest IONP concentrations caused oxidative stress in liver cells. Liver microarray analysis revealed almost 1000 DETs between the control and IONP treatment groups.	[15,215]
10	Green synthesized MgO nanoparticles (G MgO NP)	Very highly biocompatible.		Dose-dependent apoptosis in the head and tail region of zebrafish larvae with less pronounced effect in the case of G MgO NP.	[216]

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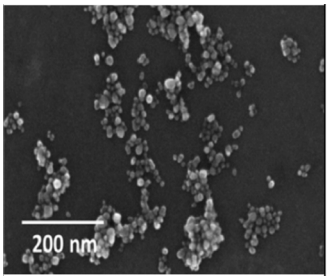
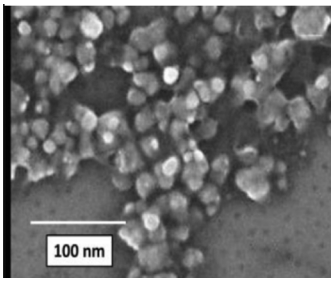
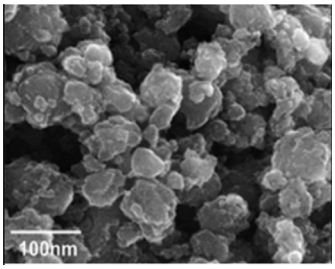
Table 4 (*continued*)

Sl. No.	Nanoparticle (NP)	Bio-compatibility	SEM/TEM/FESEM Images of NPs	Toxicity observed in Zebrafish	Reference
11	Au (Gold)NP (Green synthesis using aqueous extract of Andrographis peniculata)	Biological fabrication of AuNP can be biocompatible compared to commercially available ones.		Accumulation of AuNP leading to higher oxidative stress due to hypoxic conditions and metabolic protein interaction causing apoptosis inside the embryos.	[217]
12	Ag (Silver)NPs (green synthesized silver nanoparticles)	Lower Biocompatibility		A decrease in body weight in males was seen when exposed to it and a dose-dependent change was seen in cholesterolemia and alkaline phosphatases activity which proposed that 125 mg/kg body weight of AgNPs may harm the liver.	[218]
13	TiO_2 NPs	Highly biocompatible		Deformation in the chorion, yolk sac, heart development, notochord, tail.	[219]

9. Conclusion

Model organisms are non-human species that serve as valuable tools in laboratory research, enabling scientists to comprehend various biological processes. Among the various types of organisms being used as research models, zebrafish is very significant due to several of its attributes which add to other advantages. Zebrafish embryos possess the unique characteristic of being almost transparent, enabling us to monitor internal structure growth closely. Zebrafish eggs provide a perfect opportunity to study early developmental stages since they are fertilized and developed externally. These fish have a genetic similarity to humans of up to 70%, making them an ideal model organism. Therefore, zebrafish can be utilized to evaluate the toxic effects of potential drug candidates during the initial screening assays, occasionally employing high-throughput systems.

Toxicity profiling plays a pivotal role in the development of novel drugs and expanding the therapeutic applications of existing compounds. Toxicity assays are performed at cellular levels via in-vitro bioassays using intact organisms via in-vivo bioassays. Despite the limitations of these techniques, it is crucial to recognize that toxicity in cells or non-human species does not necessarily imply a risk to the entire organism. Nevertheless, a combined procedure of toxicity testing and chemical analysis can prove to be a powerful tool for investigating different organ toxicological studies further assessing health risks related to human beings. Omics technologies, comprising genomics, proteomics, and ecogenomics have a huge role to play in the cellular processes of an individual in a community or biodiversity in response to environmental changes with high observation throughput. Drug interactions with specific genetic variations observed prior to human trials play a vital role in organ-specific toxicities such as cardiotoxicity, neurotoxicity, hepatotoxicity, and others. Therefore, systemic studies of gene-drug interactions are conducted to better understand these effects. Toxicity profiling and its key components have been highlighted in this overview. For instance, acute toxicity, sub-acute toxicity, and chronic toxicity are three stages of toxicity screening where organisms are treated with different concentrations of the toxicant which have been deliberated in this review.

Nanotechnology is one of the most progressing technologies in the world and the application of nanomaterials in the treatment of several diseases is also implied in the model organ systems. Nanoparticles have played a major role in toxicity profiling since the accumulation of nanoparticles within the organism has been a major cause of toxicity in model systems depending on the target organ and concentration with which organisms are being. Zebrafish play a crucial role in toxicity

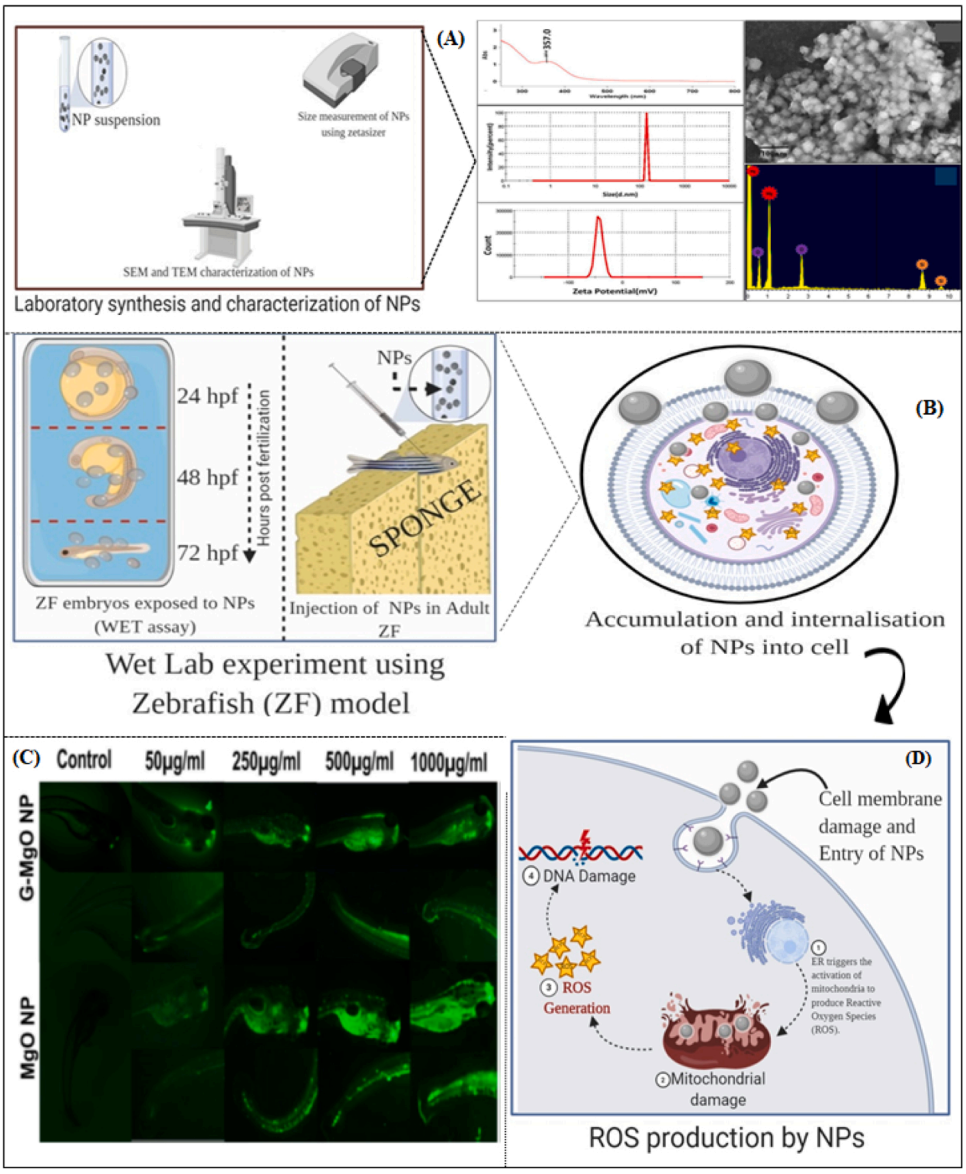


Fig. 11. (A) Physiochemical characterization of green synthesized G-MgO NP: UV-Vis spectrum showing SPR peak of MgO NP at 357 nm; The hydrodynamic diameter of G-MgO NP dispersed in HF medium; Zeta potential of G-MgO NP in HF medium; FESEM image showing the size of the nanoparticles at 100 nm scale; EDX analysis of the sample showing the elemental configuration [216]. (B) and (D) Mechanism of ROS production leading to cytotoxicity in the zebrafish model system [220,227]. (C) Apoptosis of zebrafish larvae (72hpf) exposed to different concentrations of green synthesized G-MgO NP and commercial MgO NP as determined by fluorescent image microscopy by Acridine orange (AO) staining [216].

assays, offering rapid and effortless access to valuable data on a wide range of tissues, organs, and systems. On a broader range, pre-clinical trials of deadly drugs are also carried out, so zebrafish provide an alternative source and a huge platform for biological studies. In future studies focusing on mammalian developmental and neurotoxicity, zebrafish may emerge as a valuable screening tool, potentially reducing the reliance on mammalian testing. Furthermore, with the ongoing automation of procedures, zebrafish will steadily serve as a prominent surrogate for research in neurodevelopmental biology and toxicology. Despite all these advantages, there are certain lingering drawbacks of using zebrafish as a model that has been discussed in this review for future refinement.

CRediT authorship contribution statement

Dutt Ateet: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Verma Suresh K.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chouhan Raghuraj Singh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Jerman Ivan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ghosh Aishee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Mohanty Swabhiman:** Writing – review & editing, Writing – original draft, Software, Conceptualization. **Varma Rajender S.:** Writing – review & editing, Visualization, Validation, Supervision, Formal analysis, Data curation, Conceptualization. **Patel Paritosh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation.

Kaushik Nagendra Kumar: Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mishra Yogendra Kumar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sinha Adrija:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Samal Shailesh Kumar:** Writing – review & editing, Visualization, Investigation. **Nandi Aditya:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kumari Puja:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Investigation, Data curation, Conceptualization. **Suar Mrutyunjay:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition. **Jena Snehasmita:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Singh Deobrat:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Panda Pritam Kumar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Conceptualization. **Jha Ealisha:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request by the corresponding author

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