Uttar Pradesh Journal of Zoology

Volume 45, Issue 8, Page 158-171, 2024; Article no.UPJOZ.3399
ISSN: 0256-971X (P)

A Review on Unlocking Toxicity: How *Zebrafish embryos* Help Ensure Safe Chemicals

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.56557/UPJOZ/2024/v45i84010

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/3399

Review Article

Received: 28/01/2024
Accepted: 02/04/2024
Published: 09/04/2024

ABSTRACT

Aquatic ecotoxicity testing has benefited greatly from zebrafish embryo toxicity testing (ZFET) because of its high throughput, low cost, sensitivity to a variety of toxicants, and ethical issues. This study highlights the benefits of ZFET over conventional fish toxicity testing, such as its capacity to detect teratogenic defects and possible long-term impacts. The limitations of ZFET are also addressed, notably its emphasis on acute dosages and difficulties with interspecies extrapolation. In addition to the generation of in vitro alternatives utilizing zebrafish cell lines, recent developments in ZFET technology are also highlighted, including the use of transgenic zebrafish lines and high-throughput screening techniques. The paper's conclusion includes a discussion of potential prospects for ZFET research. These include improving interspecies extrapolation methods, optimizing chronic toxicity assessment procedures, and integrating ZFET with other ecotoxicity

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testing strategies. ZFET is a great instrument for environmental protection and a crucial component in avoiding chemical contamination of aquatic ecosystems, so long as it is acknowledged for what it is and is open to future improvement

Keywords: Aquatic ecotoxicity; zebrafish embryo toxicity testing; teratogenic defects; high-throughput screening.

1. INTRODUCTION

Zebrafish embryo toxicity testing (ZFET) is one method that is becoming more and more used for determining a substance's toxicity to fish and other aquatic life. Unlike traditional research on fish toxicity, which utilize adult fish, ZFET uses zebrafish embryos at a critical stage of development. This enables researchers to subject these embryos, usually for 96 hours, to varying amounts of a test drug [1]. After this exposure, scientists carefully monitor and document the impacts on the growing embryos, keeping an eye out for any indications of mortality, aberrant development, or other growth problems. ZFET's primary benefit is its speedy identification of pollutants that may pose a threat to fish populations in our rivers [2]. This knowledge is essential for safeguarding the environment since it enables us to control and stop the discharge of pollutants [3]. Additionally, ZFET is becoming more and more popular as a more efficient and moral substitute for conventional fish toxicity testing that employs mature fish. ZFET decreases the number of animals needed for testing and lessens the harm done to them by using embryos rather than adult fish [4].

1.1 Zebrafish Maintenance

The fast reproduction, external fertilization, and clear embryos of zebrafish make them perfect for ZFET [5]. The upkeep and breeding protocols for ZFET are summarized as follows:

- **Housing:** Large aquariums (around 10–30 liters) with filtered and recirculating water are usually used to hold adult zebrafish. A certain pH (about 6.5–8.0), conductivity, and temperature range should all be maintained in the water [6].
- **Nutrition:** Since zebrafish are omnivores, they can be fed bloodworms, brine shrimp, or commercially produced flake food [7] .
- **Light Cycle**: To control zebrafish reproduction, a regular light cycle of around 14 hours of light and 10 hours of darkness must be maintained [8].

1.2 Breeding Procedures

1.2.1 Breeding Tanks

To isolate the breeding adults (ratio of 1 male to 2 female fishes), employ a barrier between separate breeding tanks. To prevent egg entrapment due to the transparency issue of the eggs, very few decorative plants are placed without compromising the natural simulated environment for the adults to breed. All the water parameters identical to the main tank should be maintained [9].

1.2.2 Breeders' Conditioning

To guarantee healthy progeny, adult zebrafish who are chosen for breeding are fed a premium diet for a few weeks.

1.2.3 Breeding

A male and female zebrafish are housed in a breeding tank together followed by removal of the barrier after maintaining the separation for the synchronized courtship process. To stop the adults from eating the eggs, a spawning mesh is positioned at the bottom of the tank [6].

1.2.4 Gathering Eggs

The adult fishes spawn usually in the early morning. For the ZFET experiment, the spawning mesh is taken off and the fertilized eggs are carefully gathered [10].

2. PREPARATION OF TEST SOLUTIONS AND EXPOSURE OF EMBRYOS

The ZFET experiment proceeds to prepare test solutions and expose the embryos after zebrafish breeding and egg collection:

2.1 Test Solution Preparation

2.1.1 Stock solution

To make a concentrated stock solution, the test material (chemical being assessed) is first dissolved or diluted in an appropriate solvent. An acetone solution, dimethyl sulfoxide (DMSO), or

fish system water are common solvents. Based on the test substance's solubility and ability to affect the embryos directly, the solvent of choice is determined [11].

2.1.2 Serial dilutions

Various test concentrations are produced by preparing a series of dilutions from the stock solution with the selected solvent. For the purpose of establishing a range of exposure levels for the embryos, this usually entails a geometric progression (e.g., ½ dilution, ¼ dilution, ⅛ dilution) [12].

2.1.3 Control solutions

Two or more control solutions are ready to go along with the test solutions. One control evaluates any possible effects of the solvent on the embryos by using simply the solvent (instead of the test drug). A different control might make use of a well-known reference toxicant, such as 3,4-dichloroaniline, for comparative analysis [13].

2.2 Embryo Exposure

2.2.1 Selection and staging

To ensure that the eggs are at a similar developmental stage, freshly fertilized eggs are carefully picked. The term "staging" describes an embryo's precise developmental stage, which is frequently indicated by the number of cell divisions or physical traits. Comparable responses between test groups are guaranteed by a consistent stage [14].

2.2.2 Transfer to exposure wells

Individual wells of a multi-well plate (such as a 24-well plate) are gently filled with 2ml of embryo medium and a healthy fertilized embryo with respective test concentration in each well i.e. 24 embryos in 24 wells each. A distinct test solution or control solution is present in every well [15].

2.2.3 Incubation

After that, the multi-well plate is set up in an incubator that keeps the water salinity ≤0.2% along with a semi-static refill of the respective test solution concentration with dissolved oxygen of ≥60% of air saturation value, temperature at 26±1ºC, pH 6.0-8.5, hardness <180mg/L CaCO3 and light cycle of around 14 hours of light and 10 hours of darkness for the growth of zebrafish embryos [16].

2.4 Factors to Consider

- Depending on the mentioned guideline ZFET methodology, a minimum of 7 embryos experimented in each test concentration, different numbers of embryos may be employed for each test concentration based on the specific research criteria.
- It is essential to use consistent handling methods to reduce embryonic stress.
- In ZFET, the exposure period is normally 48 or 96 hours, while it might be longer for certain research [17].

These procedures enable researchers to create precise test solutions and subject zebrafish eggs to various test drug doses for additional examination of possible toxicity effects.

3. OBSERVATION AND RECORDING OF ENDPOINTS IN ZFET

Following a certain time period (usually 48 or 96 hours) in which zebrafish embryos are exposed to different test solutions, scientists carefully monitor and document particular endpoints in order to evaluate the impact of the test material. These endpoints highlight possible harm brought on by the test material and act as markers of the health and development of the embryo.

3.1 Commonly Observed Endpoints

- **Mortality:** The number of dead embryos in each test concentration relative to the controls is the most fundamental outcome. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction [18].
- **Developmental Abnormalities:** Scientists keep a watchful eye out for any morphological anomalies that deviate from the expected course of development. This covers anomalies in:
- **Somite formation:** The segmented blocks that make up the muscles and backbone are called somites. Problems with development are indicated by uneven spacing or lack of somite formation.
- **Tail detachment:** The zebrafish embryo's tail separates from the yolk sac during

development. Failure to remove the tail indicates delays or disturbances in development.

- **Size and edema of the yolk sac:** The embryo receives nutrition from the yolk sac. Unusual yolk sac dimensions (excessively tiny or huge) or edema (fluid buildup) may be signs of problems with the growth or absorption of nutrients.
- **Heart function:** The survival of the embryo depends on the existence and regularity of the heartbeat. Abnormal heart rates or a lack of heartbeat indicate cardiovascular issues brought on by the test drug.
- **Additional malformations:** Scientists may notice and document additional anomalies related to development, such as defects in the eyes, fin malformations, or body curvature issues [19].

3.2 Recording Observations

- Throughout the exposure period, observations are usually taken at particular intervals (e.g., 24, 48, 96 hours). Standardized scoring methods can be employed to record the degree and occurrence of detected deviations.
- It's possible to take pictures or videos of the embryos for archiving or additional research [17].

3.3 Additional Considerations

- Depending on the study topic or the test substance's presumed method of action, different endpoints may be detected.
- To distinguish between actual anomalies brought on by the test material and changes in normal development, one must have a trained eye and specialized knowledge while seeing embryos.
- Accurate and repeatable outcomes depend on the use of consistent observation strategies and data collection procedures [20].

Through methodical observation and documentation of these endpoints, scientists may evaluate a test substance's potential toxicity to growing zebrafish eggs and mitigate its dangers to aquatic habitats.

3.4 Data Analysis and Calculation of Toxicity Values

After monitoring and documenting endpoints in ZFET, scientists analyze the data to determine critical toxicity values, which represent the test substance's overall impact on the embryos. These numbers give an exact assessment of the toxicity of the test material and enable cross-chemical comparisons [21].

3.4.1 Lethal concentration 50 (LC50)

This is the amount of a test material that, at a certain exposure duration (e.g., 96 hours), is predicted to kill 50% of the exposed embryos. Since a smaller dosage is required to kill half of the embryos, a lower LC50 suggests a more dangerous chemical.

3.4.2 No Observed Effect Concentration (NOEC)

The greatest concentration of the test drug at which no statistically significant side effects (such as death or anomalies in development) are noticed in comparison to the control group. As it represents the concentration at which no discernible harm occurs, the NOEC is seen to be a more ecologically meaningful number.

3.4.3 Lowest detected Effect Concentration (LOEC)

The concentration at which unfavorable effects are detected that are statistically significant as compared to the control group is known as the LOEC. A severe degree of toxicity may not always be represented by the LOEC, but it does serve as a baseline for possible consequences [19].

3.5 Data Analysis Techniques

3.5.1 Mortality data

Probit analysis or comparable statistical approaches are commonly used to examine mortality data from various test doses. These techniques take into consideration the variation in embryo responses when estimating the LC50 value and associated confidence range.

3.5.2 Abnormality data

To evaluate the frequency and severity of developmental abnormalities across test groups

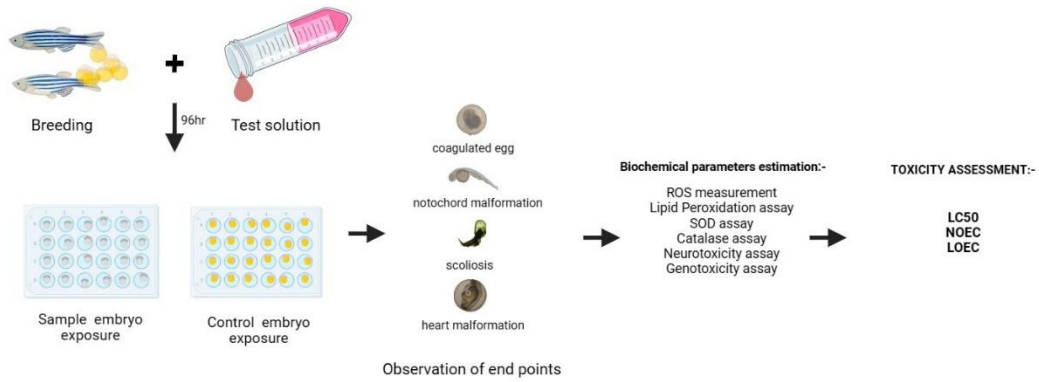

Fig. 1. Zebrafish Embryo Toxicity Test

Test solution can be a sample solution to be tested or positive control, negative control or solvent control. Full forms of the mentioned abbreviations are ROS (Reactive Oxygen Species), SOD (Super Oxide Dismutase), LC50 (Lethal Concentration 50), NOEC (No Observed Effect Concentration), and LOEC (Low Observed Effect Concentration)

and controls, statistical tests and scoring systems are frequently used in the analysis process. This aids in determining the LOEC and NOEC for impacts on development [22].

3.6 Factors to Consider

- Data analysis and the determination of toxicity levels are frequently facilitated by software applications.
- Additional toxicity estimates, such as the Effective Concentration (EC50) for certain outcomes (like heart malformation), may be computed based on the research design and observed endpoints.
- When interpreting toxicity data, it's important to take into account the particular endpoints that were evaluated as well as any potential ZFET test restrictions [23].

The possible harm that a test drug may do to growing zebrafish eggs can be measured by researchers by evaluating the observed data and computing important toxicity values such as LC50 and NOEC. This data is essential for assessing environmental risks and controlling chemical use to save aquatic life.

4. BIOCHEMICAL MARKERS ESTIMATION

According to Rombough et.al (2007), the euthanization of embryos were done by tricaine methasulfonate (MS-222) at a concentration of >1800mg/L for an hour for the biochemical estimations.

4.1 Reactive Oxygen Species Measurement

A modified version of Zhao et al.'s (2013) approach was used to evaluate the formation of ROS in treated embryos using dichlorofluorescein-diacetate (DCF-DA). Ten embryos were quickly homogenized in ice-cold phosphate buffer saline (PBS) following a 96-hour photoperiod exposure. 20 microliters of the supernatant were transferred to a tube and allowed to incubate for five minutes at room temperature after centrifugation at 12,000 g for thirty minutes at 4 °C. Following that, 8.3 µL DCF-DA (1 mg/mL) and 200 µL PBS were added, and the mixture was incubated for 30 minutes in the dark at 37 °C. A spectrophotometer (HORIVA) was used to measure the contents following the incubation time. Excitation was detected at 485 nm and emission at 520 nm. The relative fluorescence intensity served as an expression for the ROS level [24].

4.2 Lipid Peroxidation (LPO)

The high Malondialdehyde concentration reveals significant ROS levels created in that case. The Thio acid barbituric substances (TBARS) test which was published by Adeyemi et al in (2015) was used to determine that the antioxidant increased the oxidative stress of ferrous metal. In essence, it was about the utilization of exactly a hundred microliter ice-cold potassium phosphate buffer at pH 7.4 for the homogenization of about twenty embryos. When 100uL of 5% TCA

(trichloroacetic acid) has been added to the sample, it is incubated for fifteen minutes on ice. The successive materials were bled with 100 µL of 0.67 thiobarbituric acid, mixed, and spun in 2200 g for 10 minutes at 4°C. By centrifuge, the supernatant was mixed with 250 µL and then boiled. The sample's absorbance was read by a microplate reader (Synergy H1, BioTek) at 535 nm [25].

4.3 Nitric Oxide (NO)

The adjustment of the micro pestle helped to homogenize the embryos in a Tris-Hcl buffer (100 mM, pH 7.4), the product of which was the measurement of the NO. After they were centrifuged for 15 min at a speed of 4°C, 12000 g, the embryos were then transferred into 96-well plates where they were treated by mixing 50µL of Gries reagent with 50µL of supernatant. Using the microplate reader, the mixture's absorbance was read at the absorption of 543 nm as read in the instrument [26].

5. ANTIOXIDANT PARAMETERS

5.1 Reduced Glutathione (GSH)

Following the procedure that has been ascertained (Ganie et al., 2011) after the many alterations could bring the reduced levels of glutathione among the zebrafish embryo of the treated experiment. Homogenization was done by spinning at 3000 g for 10 min at 4 °C temperature. PBS (100 µL) was added to an aliquot of 20 embryos, prepared earlier. The homogenate next underwent precipitation by treating with 100 µL of 25% TCA. Then, load the mixture on 24 well plates and mix 1 mL DTNB, 60 µM, and 0.45 mL, 50 mM potassium phosphate buffer (pH 7.4). The microplate reader was set in the wavelength mode of 412 nm [26].

5.2 Superoxide Dismutase (SOD)

Marklund and Marklund have studied particular aspects of superoxide dismutase activity (1974). Medium and deionized water was used to dissolve embryos by using a micro pestle. Buffer solution with a pH 7.4 was used in the experiment before centrifuging the solution at 12000 g for 15 minutes at 4°C. A microplate reader was used to record absorbance decrease for 5 mins at 420 nm. After 50 µl of the homogenate was added to the reaction mixture (50 mM Tris-HCl buffer, pH 8.4 with 1 mM EDTA and 2.64 mM Pyrogallol) to determine the enzymatic reaction [27].

5.3 Catalase (CAT)

The procedure by that Sinha (1972) was adopted to establish the catalase activity. The embryos were later disrupted by micro pestle in 100 mM Tris-Hcl buffer (pH 7.4), furthermore they were centrifuged at 12000 g for 15 minutes at 4°C. 3 mL of reaction solution containing 5% potassium dichromate, glacial acetic acid (1:Supernatant (100 µL) was mixed with the reaction mixture that contained 10 mM phosphate buffer (pH 6.5), 10 mM MnCl2, and 100 units of ug/mL ubiquitinase. Subsequently, to the amalgamator, the mixture was put in a microplate reader at the wavelength of 570 nm which was heated for 20 min in the water bath [28].

5.4 Glutathione Peroxidase (GPx)

From Adeyemi et al. (2015) recommended strategy, glutathione peroxidase activity was calculated. A homogenizer was used to homogenize the embryos with 100 mM Tris-HCl buffer (pH 7.4) and the mixture was subsequently centrifuged at 4 °C for 15 minutes with a centrifuge that operated at a speed of 12000g. The supernatant was removed and afterwards, 880 ML of the reaction mixture (150 µM NADPH, 1 mM GSH as well as 100 mM sodium azide in potassium phosphate buffer, with pH of 7.0) was added. The absorbance of the plate was recorded for one minute using a microplate reader at 340 nm [29].

5.5 Glutathione S-Transferase (GST)

Finally, we found that the activity of GST was in the direction of increasing (Domingues et al. 2010). There were 8 embryos were used from each treatment, They were centrifuged (with relative acceleration equals 9.8m/s2 for 30 minutes at 4°C). By loading a 96-well plate with 50 µL of supernatant, the supernatant was added to it. In each well was added 100 µL of the reaction mixture (10 mM GSH and 60 mM 1-chloro 2, 4-dinitrobenzene). The absorbance of the microplate reader was taken and recorded for five minutes at the wavelength of 340 nm [30].

6. METABOLIC ENZYME ESTIMATION

6.1 Lactate Dehydrogenase (LDH)

The groups; LDH calculation was done by using the technique (2010) of Domingues et al. 9 糟 from each treatment were minced in ice in

0.1MTris-NaCl buffer at pH=7.2, and they were then centrifuged at 4 °C for 5 minutes under 3000 g. Then 300 µL of 240 µM(NADH) and 40 µL of 10 For five minutes, the absorbance read stayed 340 nm in the microplate reader using a 96-well plate [31].

6.2 Acid Phosphatase (AP)

Clemente and another two researchers (2014) employed the activity of acid phosphatase with a few method modifications. The embryos were centrifuged at 12000 g for 15 min. This was done at 4 °C. After homogenizing the embryos using a micro pestle, we used 100 mM Tris-HCl buffer (pH 7.4) to achieve this. 15 µL sodium acetate buffer (pH 5.0) and 125 µL p-nitrophenyl phosphate solution (5 mM) were put together and 10 µL of the supernatant was added to them. After shaking the liquid for 40 min at 37 °C, 150 µL of 1 M NaOH was added to the solution, and color intensity was measured at 405 nm with the help of a microplate reader [32].

6.3 Protein

Lowry et al. (1951) performed the protein assay and the results were measured in triplicate for zebrafish embryos. Through a plate reader, the absorbance of the sample was recorded at 630 nm wavelength of bovine serum albumin was used as reference [33].

7. NEUROTOXICITY

7.1 Karnovsky and Roots staining

Using the in-situ Acetyl Choline Esterase (AChE) activity assays developed by Karnovsky and Roots (1964) and Jacobson et al. (2010), the atrazine effect was measured at 72 hpf. The solution (60 mM NaC2H3O2, pH 6.4, 5 mM Na3C6H5O7, 4.7 mM CuSO4, 0.5 mM K3[Fe(CN)6], 1.7 mM AcS) was applied to the embryos exposed to the reagent, and exposed embryos were incubated. It is the browning caused by the effect of a high concentration of copper ferrocyanide that prompts us to suggest AChE activity and the embryos are rinsed gently in Buffer saline (PBS) before imaging [34].

7.2 AChE Enzyme Activity

The level of AChE enzyme activity was calculated with the method of Ellman et al. (1961). The final larvae used for the analysis were 5 larvae from a control and another group of 5 larvae that had been treated. They were homogenized with a potassium phosphate buffer of 0.1 M (pH 7.2) on ice after achieving 96 hours of growth. To obtain pure cultures free of mechanical damage, the larvae were first centrifuged at 3000 g for 4 minutes at 4 °C. Put succinctly, the absorbances were measured at 414 nm by using a microplate reader by pipetting 50 µL of the supernatant into 250 µL of the reaction solution consisting of the mixture of 1 mL 10 mM 5,5-dithiobis-2-nitrobenzoic acid solution with sodium hydrogen [35].

7.3 AO Staining

As demonstrated by Asharani et al. (2008), the AO staining was performed to put emphasis on the type of caused apoptosis in ZEA-toxicity. At 96 hours of somite's establishment, the embryos were washed with phosphate buffer saline (PBS) twice and the staining with acridine orange (5 µg/mL) solution was done for 20 minutes at room temperature. For the PBS coating once more, the embryos were finally visualized under the right inverted fluorescent microscope [36].

8. GENOTOXICITY

8.1 Comet Assay

Thirty embryos of zebrafish (n = 30) were collected as samples from the exposure of each group (n = 10) after 96 hpf for the purpose of determining DNA damage which can be done using the method described by Osterauer et al (2011). An EVOS FLc inverted fluorescence microscope (40X) of Life Technologies was employed to observe the slides and obtain images of 50 cells each. With the Comet assay software project (CASP) being used, comet pictures were analyzed and the olive tale moment (OTM) of each was determined. The outcome multiplication (OTM) refers to a combination of the DNA distance to their tails and the proportion of tails in the distribution. For far less chance of gene damage, all of the interventions were executed in the complete absence of light [37].

9. HISTOPATHOLOGY

Next, a 96-hour dark cycle followed by fixation in Bouin's solution and gradual transfer of the embryos into gas-permeable ethanol for maintenance were performed to the embryos of every treatment. The embryos were put into blocks of paraffin wax and stained with xylene

after removing the blood surrounding them. In this method, hematoxylin and eosin were utilized for staining, cutting into 5-6µm-thick slices using a microtome, and mounting on slides. Slides were studied by inverted microscopy to assess change in pathology which was the main criterion for judging the success of treatment [38].

10. BENEFITS OF ZFET OVER TRADITIONAL FISH TOXICITY TESTS

When compared to conventional fish toxicity testing that uses adult fish, zebrafish embryo toxicity testing (ZFET) has a number of benefits. Below is a summary of the main advantages:

10.1 Ethical Considerations

- **Reduced Animal Numbers**

Early in their development, zebrafish embryos are used by ZFET. Compared to conventional testing using adult fish, this greatly minimizes the number of animals needed. This is consistent with the replacement and reduction concepts found in the 3Rs (Replacement, Reduction, and Refinement) paradigm for morally acceptable animal experimentation [39].

10.2 Cost-Effectiveness and High Throughput

- **Faster and Simpler**: Compared to adult fish testing, which can take weeks or months, zebrafish embryos develop quickly, enabling tests to be completed in as little as 48–96 hours. This translates into quicker results production and lower expenses for testing facilities and animal care.
- **Smaller Test Volumes:** Because ZFET uses embryos, far less test solution volume and housing space are needed. As a result, the overall cost of test supplies and lab space is decreased.
- **Greater Testing Capacity:** ZFET tests' shorter length and smaller scale enable the testing of more compounds in less time. This high-throughput feature is useful for checking for possible toxicity in a larger variety of chemicals [39].

10.3 Sensitivity to a Wide Range of Toxic Effects

- **Early Developmental Stage**

During their quick growth, zebrafish embryos are extremely vulnerable to a variety of toxicants. Because of its sensitivity, ZFET can identify even minute changes in growth, organ development, or other critical processes, which may show harmful consequences in adult fish that are otherwise overlooked [40].

10.4 Potential for Additional Developmental and Teratogenicity Endpoints

- **Transparent Embryos:** Because zebrafish embryos are transparent, scientists may see their internal organs and development up close during the exposure phase. This makes it possible to identify birth malformations and developmental abnormalities (teratogenicity) in fish that may not be readily apparent in mature fish [41].
- **Genetic Manipulations:** To evaluate the impact of test chemicals on certain developmental pathways, researchers can make use of genetically modified zebrafish lines with specific mutations. This focused strategy offers insightful information on plausible toxicity pathways [42].

11. LIMITATIONS OF ZEBRAFISH EMBRYO TOXICITY TESTING (ZFET)

Although ZFET has several benefits, it's necessary to recognize its drawbacks in order to ensure a fair understanding of this testing technique:

11.1 Focus on Acute Toxicity and Potential Underestimation of Chronic Effects

- **Brief Exposure Period:** During the early stages of development, ZFET usually concentrates on brief exposures (48–96 hours). Although it may not fully represent the long-term impacts (chronic toxicity) that a chemical may have on fish populations with repeated or protracted exposure, this information on acute toxicity is nevertheless rather useful.
- **Limited Evaluation of Sublethal Effects**: Gross developmental defects and death are the main foci of ZFET. In this brief test, subtle, sublethal effects on immunity, behavior, or reproduction might not be easily seen [43].

11.2 Species Specificity and Extrapolation to Other Fish Populations

- **Model Organism**

Although zebrafish are a useful model organism, other fish species may not have the same physiology or chemical sensitivity. It is important to use caution when extrapolating ZFET results to other fish populations, since it could be necessary to do additional testing with ecologically relevant species [21].

11.3 Need for Well-Defined Protocols and Standardization

- **Standardization Challenges**: ZFET protocols are typically well-established, although there may be differences in breeding conditions, exposure times, and data processing techniques throughout laboratories. Variability in outcomes may arise from this, which may impede cross-study comparisons [10].
- **Substance-Specific Test Optimization:** Depending on the particular chemical being tested, ZFET methods may need to be optimized. Adjustments to the exposure regime or observed endpoints may be necessary due to factors such as water solubility, potential for bioaccumulation, and hypothesized mechanism of action [44].

12. ADDRESSING LIMITATIONS

- Researchers are working hard to come up with solutions for these constraints. This entails using multi-generational research, adding endpoints that evaluate sublethal effects, and increasing exposure durations in ZFET to account for sub-chronic effects.

- Improving laboratory uniformity and employing species-specific testing techniques where required are essential measures in guaranteeing the resilience and wider relevance of ZFET findings [45].
- ZFET may be improved as a useful instrument for environmental risk assessment and guaranteeing the security of aquatic ecosystems by recognizing and resolving these limitations.

13. DIVERSE APPLICATIONS OF ZEBRAFISH EMBRYO TOXICITY TESTING (ZFET)

ZFET is not limited to its core application in environmental hazard assessment; It is quite versatile. Here are some examples of its many applicability in different fields:

13.1 Regulatory Ecotoxicity Testing of Chemicals and Environmental Pollutants

- **Chemical Screening:** During the regulatory clearance process, ZFET is a quick and affordable way to test a variety of chemicals (pesticides, industrial effluents, and medicines) for possible aquatic life toxicity. Regulatory agencies can use this information to make well-informed judgments on the use of these substances in the environment and any possible dangers [43].
- **Environmental Monitoring:** Water samples taken from rivers, lakes, or wastewater treatment facilities can be tested for environmental contaminants' toxicity using ZFET. This offers useful information for detecting any risks to aquatic environments and putting suitable corrective measures in place [46].

13.2 Drug Discovery and Developmental Toxicology Screening

- **Drug Development in the Early phases:** ZFET is a useful tool in the early phases of drug development because to its high throughput capabilities and capacity to identify birth abnormalities, or teratogenicity. Researchers can reject inappropriate medication candidates and promote safer choices for continued development by identifying possible developmental toxicity early on.
- ZFET can be employed by researchers to get an understanding of the precise processes via which medications or other chemicals lead to developmental defects. The development of safer medications and more effective treatment plans may depend on this knowledge.

13.3 Environmental Monitoring and Water Quality Assessment

- **Biomonitoring Tool:** ZFET is a biomonitoring tool that may be used to evaluate aquatic ecosystems' general health. Researchers can find continuing challenges to fish populations and possible pollution by exposing zebrafish eggs to water samples from several sites [47].
- **Water Quality Assessment:** For a variety of uses, such as aquaculture and water treatment facilities, ZFET provides a sensitive and quick method of evaluating water quality. Finding even trace amounts of toxicants contributes to the security of fish utilized in research settings or meant for human consumption [18].

13.4 Genetic and Developmental Studies Using Zebrafish Embryos

- **Modeling Human Diseases:** Zebrafish embryos are useful models for researching human developmental diseases because of their high degree of genetic conservation with humans. ZFET may be used by researchers to examine how certain genetic mutations or environmental exposures affect early development. This can provide light on the origins of birth abnormalities and provide possible treatment approaches.
- **Gaining Knowledge of Developmental Processes:** Zebrafish embryos are perfect for investigating the many mechanisms involved in normal growth because of their transparency and quick growth. Genes and signaling pathways essential for organ creation and general embryonic health can be identified using ZFET [22].

14. RECENT ADVANCEMENTS IN ZFET TECHNOLOGY

Researchers are continually coming up with novel methods to improve the capabilities of zebrafish embryo toxicity testing (ZFET) and overcome its limitations. An examination of a few encouraging recent developments is provided below:

14.1 Incorporation of Transgenic Zebrafish Lines

- **Enhanced Specificity and Sensitivity**: Conventional ZFET is based on broad endpoints such as deformities and death. Researchers may customize the test to identify certain forms of toxicity by utilizing transgenic zebrafish lines that have specific genetic changes.

As an illustration, consider the following:

- Fluorescent marker lines in certain organs allow one to see how those organs are affected when exposed to a test material [48].
- The ability of a chemical to obstruct certain toxicity pathways may be determined by looking at lines with mutations in genes known to be implicated in those processes.

14.2 Integration of High-Throughput Screening Methods

- **Quicker and More Thorough Analysis:** Traditionally, ZFET has relied on endpoint scoring and manual observation. Higher throughput screening techniques, like as automated image analysis and microfluidics, can be integrated to process more embryos more quickly and analyze developmental characteristics more thoroughly [49].

14.3 As an illustration, consider the following

- Numerous embryos exposed to various test doses can have their morphological anomalies quickly quantified by automated image analysis tools.
- By using microfluidic devices, researchers may replicate real-world situations with variable pollutant concentrations by creating complicated exposure profiles for the embryos [50].

14.4 Development of *In vitro* Alternatives Using Zebrafish Cell Lines

- **Ethical Considerations and Lower Costs:** Zebrafish cell line-based in vitro alternatives provide a possible improvement over ZFET by lowering the quantity of embryos needed for testing. These cell lines can be engineered to react a certain way to particular poisonous insults [51].
- **Present Status:** Although encouraging, in vitro zebrafish cell tests are still in the early stages of development and need additional

verification to guarantee that they faithfully capture the nuanced reactions seen in whole embryos [52].

15. CONCLUSION

Testing for zebrafish embryo toxicity (ZFET) has become a useful method for determining if a chemical poses a risk to aquatic life. It is a major advancement over conventional fish toxicity testing due to its cost-effectiveness, sensitivity to a wide range of harmful effects, high throughput, and ethical issues. ZFET gives researchers the ability to pinpoint substances that may be harmful to fish populations at a key stage of development, which is vital information for attempts to safeguard the environment.

Ongoing studies have identified several of ZFET's shortcomings, mostly related to its emphasis on acute toxicity and difficulties in generalizing findings to other fish species. Scholars are presently devising tactics to tackle these constraints. This entails improving interspecies extrapolation techniques, streamlining chronic toxicity assessment methodologies, and combining ZFET with additional ecotoxicity testing approaches.

Additionally, ZFET technological improvements including the use of transgenic zebrafish lines and high-throughput screening techniques offer improved sensitivity, efficiency, and the capacity to identify certain toxicity types. Zebrafish cell lines have been used to produce in vitro substitutes, which presents opportunities for improvement and ethical problems. To sum up, ZFET is an effective and dynamic instrument for assessing aquatic toxicity. ZFET will continue to be essential in guaranteeing the security and well-being of our aquatic ecosystems as long as it recognizes its limitations and actively pursues future growth.

16. FUTURE DIRECTIONS

Even though ZFET is a potent method for determining aquatic toxicity, research is still being done to overcome some of its shortcomings and expand on its potential. This is a sneak peek at several important directions for future ZFET research:

16.1 Refining Protocols for Chronic Toxicity Assessment

- **Lengthening Exposure Durations:** The majority of ZFET protocols in use today concentrate on brief exposures (48–96 hours). Subsequent investigations will look at extending exposure times to account for sub-chronic impacts (weeks to months), which may be important in determining the long-term effects of chemical exposure on fish populations [53].
- **Multi-generational Studies:** Research involving the exposure of several generations of zebrafish to a test drug can shed light on the effects of the substance over generations as well as its consequences on population health [29].
- **Including Biochemical and Physiological Endpoints:** Future ZFET research may include additional studies like as enzyme activity, stress hormone levels or gene expression to offer a more complete picture of longterm toxicity, even if death and malformations are still important endpoints.

16.2 Addressing Interspecies Extrapolation Challenges

- **Species-Specific Testing Techniques:** Although zebrafish are a useful model, it's important to recognize interspecies distinctions. To increase the ecological significance of the results, future studies may combine ZFET testing with ecologically significant fish species [54].
- **Comparative toxicology studies:** These studies may be used to find possible trends and improve techniques of extrapolation by evaluating the toxicity of substances across several fish species with differing life cycle features and environmental sensitivity [45].
- **Modeling Techniques:** The prediction of impacts on fish populations in the environment can be enhanced by using computer models that include ZFET data with ecological and physiological data from different fish species [21].

16.3 Integrating ZFET with Other Ecotoxicity Testing Methods

- **Tiered Testing Approach:** ZFET is a quick and affordable first screening step that may be included into a tiered testing plan. More intricate testing with adult fish or whole ecosystems can be carried out for

a more thorough risk assessment if a chemical exhibits possible toxicity in ZFET [43].

- **Combining in vitro and in vivo methods:** Zebrafish cell tests conducted in vitro show potential for effective preliminary screening. In order to build a more reliable testing battery that capitalizes on the advantages of both strategies, future research may investigate merging these assays with ZFET [41].
- **Handling Environmental combinations:** Complex combinations of pollutants are frequently seen in aquatic habitats found in the real world. Prospective studies on ZFET may investigate the exposure of embryos to chemical combinations in order to gain a better understanding of possible interactions and cumulative effects.

ZFET has the potential to develop into an even more formidable instrument for environmental preservation by aggressively exploring these future paths. enhanced interspecies extrapolation techniques, enhanced chronic toxicity assessment processes, and integration with other ecotoxicity testing tools will guarantee that ZFET stays at the forefront of protecting aquatic ecosystems from any chemical pollution dangers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://prh.mbimph.com/review-history/3399