



Zebrafish, *Danio Rerio* as a Replacement Alternative Model Useful in CKDu Experiments

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Abstract. Zebrafish (*Danio rerio*) and its embryo has become a popular replacement alternative among the scientists because of many scientific attributes. As it is a model commonly used in ecotoxicology, our plan is to use this model to identify causative factors leading to chronic renal disease of unknown origin prevailing among poor, farming communities in Sri Lanka. This paper describes briefly the training underwent at University of Antwerp, Belgium and how zebrafish model could be used to address an important public health issue in Sri Lanka.

Keywords: Zebrafish · *Danio rerio* · Replacement alternative models
CKDu

Introduction

The Concept of Replacement Alternative

Interest of scientists has been deviating since 20th century, from the use of animals in their experimental work towards substituting with ‘Alternatives’, thus reducing the use of live animals in experiments. This ‘Alternative’ concept is principally the ‘Replacement’ alternative that was indicated in the book; ‘The Principles of Humane Experimental Technique’ written by Russell and Burch in 1959. Although many methods of replacement have been developed and used by researchers, most of these are not **absolute** replacement models. As absolute replacement models should not involve whole animals and animal tissues, in many instances models used by researchers are **relative**. Relative replacement models include lower vertebrates, invertebrates or animals having lower level of sentience and tissues, cells, sera and embryos etc. of animal origin. These relative replacement models of course reduce or prevent the use of conscious living vertebrates [1].

Scientific Importance of Zebrafish (*Danio Rerio*)

Among the accepted relative replacement models, the zebrafish (ZF) and its embryo model have been of interest to the researchers due to its wide spectrum of scientific applicability. ZF and its embryo have been used in diverse fields of science including

developmental biology, oncology, pharmacology, toxicology, teratology, genetics, neurobiology, environmental sciences, stem cell research etc. [2–6]. Identification of substances/key molecules responsible for regenerative capacity of damaged heart muscle, retinal tissues of eyes, nerve fibers shed light for the scientists one day to focus on new therapies for people with ischaemic heart disease, spinal cord injuries and to combat degenerative eye disease damage in humans [7–9].

Scientific Attributes of Zebrafish

ZF, specially its embryo model has its wide acceptance and popularity as a replacement model due to many scientific attributes such as small size of ZF, ease of maintenance, low cost, rapid growth rate, high fecundity rate, external fertilization, optical transparency of the embryo (Figs. 1 and 2), ease of genetic manipulations, high genetic similarity to humans and regenerative capacity [2, 5].

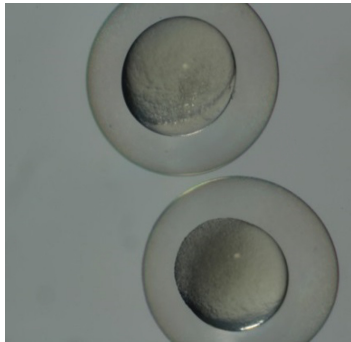


Fig. 1. Normal embryos after collection (Inverted microscope-Leica 10447137 model 10X/23, X1.0)

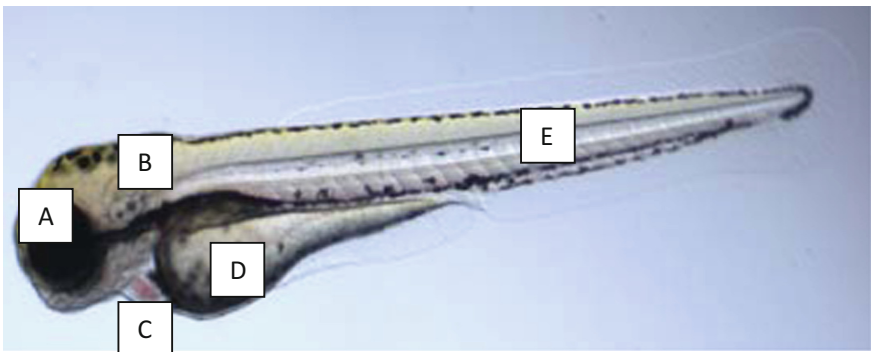


Fig. 2. Normal Zebrafish embryo at 72 hpf. (Inverted microscope-Leica 10447137 model 10X/23, X2.0). A: Eyes, B: Otoliths, C: Heart, D: Yolk sac and its extension, E: Vertebral column

Introduction of Zebrafish Model to Sri Lanka

Scientific benefits of ZF and its embryo was unknown to most of Sri Lankan researchers until its introduction to Sri Lankans at the Inaugural Scientific Conference of the Sri Lanka Association for Laboratory Animal Science (SLALAS) in January 2014 by Dr. Francois Busquet, CAAT-Europe Policy Coordinator, University of Konstanz, Germany. ZF, which is a native species in Sri Lanka, is used as an ornamental fish [10]. The wild type and ZF in different colours, produced through genetic manipulations by the breeders, are available in Sri Lankan aquaria for this purpose.

Materials and Methods

Comprehensive Training on Zebrafish Model

Transparency of the developing ZF embryo during demonstrations at the Inaugural Conference of SLALAS was very impressive and an eye opener for most of Sri Lankan researchers to concentrate on replacement alternatives. This created an interest for acquisition of more knowledge and skills on ZF embryo model. Thus a 2-week comprehensive training at the ZF lab in University of Antwerp, Belgium was possible because of the Overseas Special Training Fellowship granted by the National Science Foundation of Sri Lanka. The material such as ZF embryos, testing chemicals, inverted microscopic facilities with recording of images etc. needed during training was kindly provided by Prof. Dries Knapen, Head of the ZF lab of University of Antwerp through the research grants secured by him.

During this 2-week training, the principal focus was on three areas.

1. Operation and daily maintenance of the ZF housing facility provided an insight in to different housing systems; fully automated standalone, semi-automated tanking and aquarium type housing systems that could be established in a ZF lab depending on the financial capacities. Hands-on was possible on the frequency and method of water quality testing for pH, temperature, salinity/conductivity and hardness of water, which are prerequisites for reproduction, and maintenance of fish in the tanks. It was also emphasized the need for different types of filters; chemical, biological and UV. Maintenance of good standards improves quality of research procedures and reproducibility of research data.
2. Daily maintenance and reproduction of ZF in practice covered nutritional requirements of ZF at different stages of growth, feeding patterns and need for variation in food types. In order to use ZF embryo as a research model, knowledge on how to facilitate spawning, collection of eggs, selection of good quality eggs are essential, and thus practiced.
3. Standardized morphological scoring of ZF embryos and larvae as an important item during training gave an insight in to how ZF embryo could be used for water quality testing and acute toxicity testing of substances according to ISO 15088:2007 and OECD guideline 236 [11–13]. Two concentrations; 600 mM and 1200 mM of caffeine (Kofeina, 1, 3, 7-Trimethylxanthine, Sigma Aldrich) were used during practice. When performing this test, newly fertilized ZF eggs (n = 60 per sample

solution) in water samples different concentrations of toxic substances for a period of 96 h (96 h post fertilization—96 hpf), with positive (3, 4-Dichlore aniline) and negative control solutions should be incubated. Observations with an inverted microscope at every 24 h are needed to identify the four endpoints; coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac (Figs. 3, 4 and 5), lack of heartbeat. At the end of the exposure period, determination of acute toxicity could be done based on a positive outcome in any of the above mentioned recorded observations leading to calculation of LC50 value.

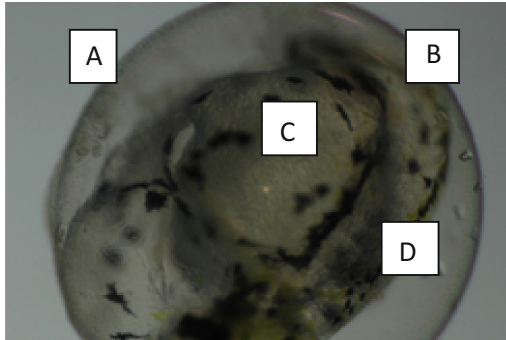


Fig. 3. At 72 hpf of exposure to 1200 mM of caffeine Solution. (Inverted microscope-Leica 10447137 model 10X/23, X3.0). A: Embryo is not hatched, B: Non detachment of the tail, C: Presence of severe edema in the developing embryo and D: Lack of somite formation

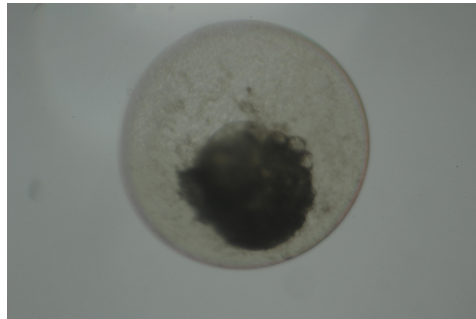


Fig. 4. Coagulated embryo (Inverted microscope-Leica 10447137 model 10X/23, X1.0)

Results

In addition to four end-points of acute toxicity test, abnormalities such as malformation of tail, pectoral fin, yolk sac, head, eyes, otoliths, mouth and heart; pericardial and yolk sac edema, accumulation of blood, disturbed or no blood flow in the tail; abnormal pigmentation; non-detachment of tail and un-inflated swim bladder leading to abnormal

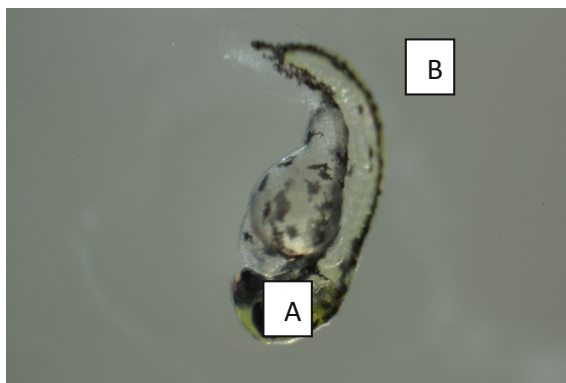


Fig. 5. At 72 hpf- Abnormal larvae. (Inverted microscope-Leica 10447137 model 10X/23, X1.0) A: Percardial edema, B: Curved tail.

movements of larvae, were also observed during this training. Necessity for adoption of a scoring system to quantify observations for scientific presentation of data was emphasized.

When using ZF model in experimental procedures the need for ethics and welfare aspects too need to be considered. It is accepted that ZF larvae develops the capability in independent feeding without depending on the food supply from the yolk sac and feeling of pain sensation around 120 hpf (Drs. An Hagenaaers and Lucia Vergauwen of Zebrafish lab, University of Antwerp- Personnel communication during training, [14]). Therefore, the need for ethics approval for the use of ZF embryo in acute toxicity testing does not arise.

Discussion and Conclusion

Acute toxicity test with ZF embryo as a replacement alternative has become the easiest and most convenient test because the test could be performed within 4 days of post fertilization (96 hpf) without breaching ethics and welfare. However, there are several tests where ZF can be used subjected to ethics approval. ZF model has a wide spectrum of applicability in the Sri Lankan context instead of rodents and rabbits thus the application of 3Rs concept in the experimental procedures.

Scientific Applicability of Zebrafish Model in the Sri Lankan Context

Being a fresh water fish, ZF and its developing stages are sensitive to changes in their immediate environment [15]. These changes could cause mortality, and also affects all their activities and growth in developing stages. Therefore, ZF model could be used as a replacement alternative for water quality testing in order to address one of the long-standing problems of public health concern, 'Chronic Kidney Disease of unknown origin' (CKDu) in Sri Lanka.

CKDu is said to be multifactorial in origin leading to hospitalization of over 1,100 CKDu patients per month in Sri Lanka and 300 deaths per year while the first patient

was identified in 1994. Total number of patients in Sri Lanka exceeds 70,000. This CKDu problem is prevailing among farming communities and it's a condition, which is slowly progressing and becomes irreversible. Moreover it is asymptomatic until last stages producing mainly tubule-interstitial fibrosis and tubular atrophy as evident in renal biopsies of affected patients [16, 17].

In this context the whole fish as well as its embryo and larvae as per OECD guidelines 236, 203, 210 and 215 could be used to check the effect of suspected heavy metals leading to CKDu and water samples obtained from all sources of water in the endemic areas compared with that of non-endemic areas, as well as with laboratory reconstituted water controls according to the following plan.

1. Collection of water samples from CKDu endemic and non-endemic areas
2. Performance of tests with
 - collected water samples
 - filtered water samples using specially developed filters
 - suspected heavy metals in different concentrations
3. Histopathology with H&E for renal effects using the whole embryo and harvested kidneys of adult fish.

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