Zebrafish: A cost-effective model for high-throughput toxicity screening

HANISAH AZHARI

ABSTRACT

Zebrafish is a highly potential model to be used in high-throughput toxicity screening of a compound. The flexibility in handling the fish, the embryos and larvae allows the screening to be completed in a shorter duration. The use of zebrafish embryo can cut the costs involved in traditional toxicity studies involving *in vitro* and *in vivo* mice studies. It also adheres to the basic 3R principle in animal ethics, which involved "replace, reduce and refine". This mini review will discuss on the basic information of using zebrafish embryo and larvae as a tool to study the toxicity of a studied compounds in a more cost-effective way.

Key words: zebrafish, toxicity, screening, embryo, larvae

ABSTRAK

Zebrafish adalah model yang sangat berpotensi untuk digunakan dalam penyaringan ketoksikan tinggi di dalam formulasi. Kemudahan dalam menangani ikan zebrafish, larva dan embrio memudahkan proses penyaringan ketoksikan dalam tempoh yang lebih singkat. Penggunaan embrio zebrafish juga boleh mengurangkan kos yang terlibat dalam kajian ketoksikan yang biasa dijalankan, iaitu secara "in vitro" dan "in vivo". Ia juga mematuhi prinsip asas 3R dalam etika penyelidikan haiwan yang melibatkan "penggantian, pengurangan dan memperbaiki" kaedah kajian. Penulisan ini akan membincangkan maklumat asas menggunakan embrio zebrafish dan larva sebagai alat untuk mengkaji ketoksikan formulasi dengan cara yang lebih kos efektif.

Kata kunci: zebrafish, toksisiti, penyaringan, embrio, larva

INTRODUCTION

The study of toxicity of molecules in health sciences area has become a major request. The discovery of new molecules requires a well conducted toxicity studies before they can be marketed. Old molecules or commonly used compounds are also being tested and monitored for their acute and chronic toxicity effects. It is very crucial to ensure the compounds did not pose any threat to the human either after short term or long-term exposure. The toxicity effects can be measured in term of chronic or acute damage.

In different areas of health sciences, compounds that are commonly tested for toxicity can be from food ingredients, drug molecules, drug excipients, nanoparticle development, pharmacological compound and lots more (Lantz-Mcpick et al., 2015). The compounds or formulations are commonly tested in the initial stage of product development or as the post monitoring surveillance to ensure its safety (Ribeiro et al., 2015). *In vitro* cell studies are commonly used as first line test for toxicity studies. Tests like MTT assay is commonly used to evaluate the level of toxicity from the compound or to measure the toxic concentration (Tolosa et al., 2015). Flow cytometry is another cell-based method that can be used to study the toxicity effect of the compounds (Suman et al., 2015).

After the initial study with the cell-based model, the compounds usually tested in *in vivo* mammalian model using mice model (Pugazhendhi et al., 2018). In general, animal study requires a complicated lengthy ethics' approval with high costs. The basic principles in animal ethics include replace, reduce and refine (Tornqvist et al., 2014). In the first step, researchers should consider replacing the animals with an alternative option (Otto et al., 2018). Depending on the area of study, alternative study methods should be considered and avoid using live animal or avoid using bigger animals. For example, tissue culture or perfused organs can be used alternatively to using live animal (Hampshire et al., 2019). The alternative method can be cost-effective and time saving. It also provides enough level of knowledge and evidence to the studied area. A strong justification needs to be considered before deciding to use an animal model, when a non-animal model can be used to answer the same research question.

However, if there is a strong need to use the animal, then "reduce" comes into place. The number of animals to be used need to be kept at minimum (Aske et al., 2017). The researcher needs to consider the minimum number of

animals which can give them sufficient scientific evidence to support their work. Studies should not be repeated unnecessarily, which will increase the number of animals used. It should be enough to provide statistically significant answer, without the need to sacrifice a large number of animals. And lastly, the experimental procedures must be refined to reduce the suffering of the animal. Refinement of the process is important to reduce the severity of "inhumane" procedures applied on the animal. The condition and wellbeing of the animal need to be carefully evaluated to protect the welfare of the studied animal. This include all the processes involved from the husbandry and housing, up to the experimental procedure and process of recovery (Cheluvappa et al., 2017). Toxicity studies can cause suffering and pain to the animal and the procedures involved must consider the welfare of the animal. In certain studies that are conducted to determine a toxic dose to the animal, it is crucial to practice the 3R principles. A more cost-effective model is needed to reduce the dependence on mammalian model in the initial toxicity screening studies. To comply with the 3R principles in animal studies in toxicity studies, using zebrafish model can offer a huge advantage in the initial stage of the research work. Since most of the initial stage of *in vivo* work starts with mice model, zebrafish can be a suitable replacement model to bridge the information from *in vitro* studies to *in vivo* studies.

ZEBRAFISH TO REPLACE MICE MODEL

Zebrafish (*Danio rerio*) originates from Southeast Asia and have been widely utilised in various field of studies, especially in molecular genetics and developmental biology (Langheinrich, 2003 & Kawakami et. al., 2016). Zebrafish have been reported to be 200 to 1000 times cheaper than using mammalian models and the cost can be as low as 1% of that of rodents (Kalueff et al., 2014).

Zebrafish offer several advantages as an animal model as compared to mice model. It has been reported that 70% of their genes are similar to those in human and they can therefore be a suitable *in vivo* model for certain genetic or developmental studies (Kalueff et al., 2014 & Lepanto 2019). There are various advantages of using zebrafish model over mice model in drug development studies. Zebrafish are a small size vertebrate and only need a small space for living. The husbandry of zebrafish is simple where a single fish tank can accommodate about 50 to 60 adult zebrafish while the larvae and embryo can live in a minimum of 50 uL of liquid (Kari et al., 2007). In contrast, fewer than 10 mice can live together in a cage. Zebrafish have a fast reproduction rate with about a 10 days breeding cycle and they produce from 50 to 300 eggs at a time, while mice can produce around 10 pups in a single breeding. Zebrafish will take about 24 hours after fertilisation to develop into larvae, while the gestation period for mice is about 21 days (Augustine-Rauch et al., 2010).

The large number of offspring and shorter gestation period will significantly reduce the maintenance cost and offers a better option for high throughput screening of high cost molecules (Bowman et al., 2010). Furthermore, the larvae are about 3 to 8 mm length and this allows experiment to be conducted in 96 well plates using adapted cell culture based method (McGrath et al., 2013).

Zebrafish also have external fertilisation where the embryo survives and grows outside the body, while in mice, have placental viviparity where the pups grow inside the mother until birth. In zebrafish, manipulations during the gestational period can be conducted by treating the embryo after fertilisation and development can be observed through the transparent skin (Augustine-Rauch et al., 2010). The transparency allows live imaging to be conducted without the need to sacrifice or dissect the animal (Zon et al., 2005).

Organogenesis begins as early as 24 h.p.f (hours post fertilisation) with organs being fully developed at about 5 d.p.f. (days post fertilisation) (de Esch et al., 2012). Zebrafish also has short life stages before it develops into adult fish, while it has a longer life span (as an adult) than mice (Kalueff et al., 2014). The stages of zebrafish life are summarised Table 1.

ZEBRAFISH EMBRYO IN TOXICITY SCREENING

Adult zebrafish can produce hundreds of larvae in single mating and this will allow high-throughput toxicity screening for studied compounds (Dockins et. Al., 2019) In toxicity screening using zebrafish, the embryo is commonly used to measure the toxicity level of the compound and to observe the acute and chronic toxicity effect (Zhang et. Al., 2018). Initially, adult zebrafish were kept in pairs in a breeding container. A separator is inserted between the zebrafish to control their mating time. The zebrafish pairs will be kept overnight in the same container but separated. This is to accustom the fish with their

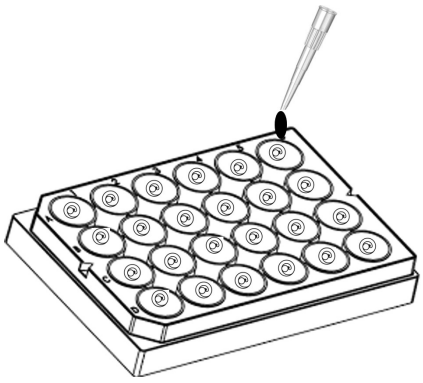
partners to maximise the chance of fertilisation. Early morning is the most suitable time for mating. The separator will be removed early in the morning and the pairs will be left to mate (Brady et. Al., 2016).

Table 1. Stages of zebrafish life from fertilisation until death (h.p.f: hours post fertilisation, d.p.f: days post fertilisation) (Spence et al., 2008).

Age	**Stage**
0 to 72 h.p.f	Pre-hatching (embryo)
72 to 120 h.p.f	Post-hatching
1 to 29 d.p.f	Larvae
	Start swimming at 5 d.p.f
30 to 89 d.p.f	Juvenile
90 d.p.f to 2 years	Adult
	Sexually active after 90 to 120 d.p.f
> 2 years	Aged fish (to study aging)
4 to 5 year old	Death

Once the eggs are fertilised, they are collected, and only fertilised eggs were chosen. The eggs can be studied for toxicity at a different stage of life. At 0 to 72 hours post fertilisation, this stage is called the pre-hatching stage (embryo) and this is a suitable time to do the toxicity studies (Pohl et. Al., 2019). The embryos are divided into small well plates and the compound (in a water-based solution) will be added into each well at the studied concentration (Figure 1). The embryos will be incubated with the compound at a selected time frame and the number of surviving embryos will be recorded.

Figure 1. 24 well plate is filled with zebrafish embryo and treated with studied compound.



The toxicity level of the studied compound to the embryos can be captured using light microscope and fluorescence microscope. Visual observation under the normal light using upright microscope will allow initial observation on the toxicity level of surviving embryos. Non-surviving embryos can be observed under light microscope from the physical appearance of the embryo. In order to get a more in-depth result, acridine orange or TUNEL assay can be used to evaluate the level of toxicity (apoptosis and necrosis). The result can be analysed qualitatively from the number of dead cells in the embryos, or it can be analysed quantitatively using fluorescence intensity of the staining agent (Asharani et al., 2008 & Usenko et al., 2007).

To study the toxic effect in an acute setting, visual observation on surviving cells and embryo can provide the information after the embryo is incubated in specific time and the images were taken straight after incubation time. However, to investigate the long-term chronic effect from the exposure to the compound, the compound was removed from the embryo (rinse off) and the embryo was left to their normal environment. The embryo is allowed to live and

grow for a period of time and the development of each organ was observed. This method is used to measure the chronic effect of the compound on the organ development. The embryo will grow into transparent larvae, where the growth of the larvae and the organ development can be observed under the light microscope. Any toxic damage to the embryo will affect the development of certain organ and possibly at different concentration of the compound, the extent of the toxic effect will differ as well. These changes can be easily observed from each larvae and can be recorded and published as qualitative data.

ADVANTAGES AND LIMITATIONS IN USING ZEBRAFISH MODEL FOR TOXICITY SCREENING

Due to the low cost and compliance in ethics principal, zebrafish model can offer a huge advantage in providing toxicity information of the studied compound. Even though zebrafish study unable to replace the importance of using mammals, it can bridge the information between *in vitro* and *in vivo* studies. The fact that *in vitro* studies can be done in a large scale, the cost involved in typical *in vitro* setting can high and while the results might not be translated into animal model. There are limitations in using cell-based model for toxicity studies and these include reproducibility in different cell lines used. Different cell lines will behave differently on the toxic effects of the compound. The results cannot be replicated with different cell lines and this is a big challenge for the researcher to understand the extend of toxic effects of their compounds.

However, when zebrafish model is used, it can represent the real toxic effect of studied compounds to living animal due to their organs having functional similarity to human (Bradford et. al., 2017). Even though it cannot replace the mammmalian model, it can bridge the gap between *in vitro* and *in vivo* data.

The large number of embryos produced by a pair of adult fish will allow a large-scale toxicity studies to be conducted. When researchers use mice model, the number of animals usually very limited and it also limits the capacity to obtain more information on the toxicity. By using zebrafish model before moving to mice model, researchers can do a large-scale toxicity screening before selecting the proper compound or concentration to be used in mice model (Yang et al., 2018). This will give more confidence and data on the process of using the right compound for further studies.

Even though zebrafish has organs with functional similarity to mammals. However, it does not have lungs. Zebrafish uses gills to breathe and get oxygen into their blood stream. And because of this, zebrafish model is not suitable for studies related to lungs.

CONCLUSION

Zebrafish is generally a new animal model currently used in developmental studies and in toxicity screening. It is widely accepted in various areas as a highly potential model to provide various type of data to the scientists. Even though the model is not established enough as compared to mice model, zebrafish can still provide a tremendous amount of data at a fraction of a cost compared to mice model.

In toxicity screening studies, even though zebrafish cannot fully replace mammalian model, it can supplement the lack of information obtained in cell culture studies. By doing high-throughput toxicity screening in zebrafish larvae or embryos, more toxicity information will be available to researchers before they can move to mice model. This will cut the cost involved in mice study and adhere to the 3R principle in animal ethics.

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HANISAH AZHARI*

Faculty of Pharmacy,
National University of Malaysia

*Corresponding author: hanisahazhari@ukm.edu.my