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Review

Zebrafish as Toxicological Model for Screening and Recapitulate Human Diseases

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Abstract: Embryonic and larval *Danio rerio* is increasingly used as a toxicological model to conduct rapid in vivo tests and developmental toxicity assays; the zebrafish features as high genetic homology to mammals; robust phenotypes; and its value in high-throughput genetic and chemical screening have made it a powerful tool to evaluate *in vivo* toxicity. New methodologies of genome editing as CRISPR/Cas9; ZFN or Talen make it a suitable model to perform studies to pair human genetic diseases as well. This review surveys recent studies; employing zebrafish as experimental model; comparing it with other *in vivo* and *in vitro* models; presenting zebrafish as a potent vertebrate tool to evaluate drug toxicity *to* facilitate more extensive; easy and comprehensive knowledge of new generation drugs.

Keywords: zebrafish; models; evaluation; drugs; cardiotoxicity; genotoxicity

1. Introduction

In drug discovery, cardiotoxicity is one of the major concerns for pharmaceutical companies, being a common, unfavorable complication associated with drugs used in oncological (Hong, 2010,), neurological (Schug, 2006) or other treatments (Redfern, 2003). This fact has become an important issue, with particular relevance for children and adolescents, as they may be more susceptible to toxic effects, and often use these treatments off-label (Ramjaun, 2015).

Many drugs (belonging to different chemical and pharmacological groups) can affect ionic channels, associated with the potential for QT interval prolongation in the heart's electric cycle, leading to ionic channel blockade in the cardiomyocyte membrane (Redfern, 2003). Those events are linked to a higher risk of TdP (torsade de pointes) being a very complex process to accurately predict its scale. This fact is one of the most important outcomes of cardiotoxicity assessment of new molecules (Fradley, 2015), together with the reduction in hERG currents, derived to adverse drug effects (human ether-a-go-go-related gene). Those are undesirable side effects of non-antiarrhythmic compounds and it has prompted the withdrawal of several blockbuster drugs from the market (Thomas, 2006), making necessary studies on mechanisms of hERG channel inhibition, providing significant insights into the molecular factors that determine state-, voltage-, and use-dependency of hERG current block (Thomas, 2006). Biet and colleagues, 2015, correlate also the increased expression of neuronal sodium channels within the heart to epilepsy-related cardiac arrhythmias associated with QT prolongation on the electrocardiogram. Then a better understanding of channels as hERG and neuonal sodium channels, could improve treatment that develop side effects on cardiac repolarization. Using zebrafish embryos, Langheinrich and colleagues (2003) reported that embryos express an orthologue hERG, named zERG, affected toward a range of QT-prolonging drugs, inducing severe arrhythmia. Then *In vivo* studies as presented by Langheinrich et al., (2003), represent an essential step in drug development and toxicity studies, as current requirements are high and include *in vivo* and *in vitro* assays to increase drug efficacy, minimizing toxicity.

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Despite superior animals have been for many year models of excellence used to evaluate drugs toxicity, population's pressure seeks, theirs reduction. The zebrafish presents itself as a reliable vertebrate model to evaluate, developmental toxicity, general toxicity and to make an initial drug screening. Derived from its use, have been reported comparable results to the obtained with higher vertebrates model. (Ducharme, 2014).

This manuscript surveys recent studies testing the toxicity of certain drugs used to treat human malignances, using diverse animal models and resume the use of the fish in drug screening and mentions, zebrafish as a small vertebrate to study rare disease suitable for genetic manipulation (Garcia, 2016).

2. Zebrafish as cardiotoxicological tool

Although physiologically differences are evident between zebrafish heart and superior animals, the zebrafish has become a good option to study heart development, supported by Bartman, 2004, and even for heart regeneration (Poss, 2002). The zebrafish has contributed to obtain measurements as action potential trough voltage mapping, to determine cells coupling (Panáková, 2010), and this fact together with calcium signaling, are important for cardiomyocyte proliferation and differentiation (Andersen, 2015).

During many years, large animals, such as mice, rats and rabbits, have been widely used to study cardiotoxicity after drug administration (Guenancia, 2016, Vasilaki, 2015, Lamore, 2013), presenting some limitations. For instance, rodents can be insensitive, particularly when endpoint measurement is based on the left ventricular contractile function (Chu, 2007). This may be due to rodents' ability to compensate loss of myocytes by recruiting alternative mechanisms.

According to the United States government, rodent and rabbit toxicity testing has been the standard for assessing acute toxicity since the 1950s. However, the process is costly and time consuming, which has led to a backlog in chemical testing (National Research Council. The National Academies Press; 2007). Because of these limitations, it has increased the need of other animal models as alternatives.

The zebrafish is particularly suitable for this purpose because it represents a vertebrate species, its genome has been sequenced, and a large number of synchronously developing, transparent embryos can be produced. In particular, the zebrafish is known to give good value with respect to cost and time, and has become an important tool to evaluate Geno-cardiotoxicity, to study embryo development and general toxicity. For instance, several compound screens, including some evaluating drug-induced cardiotoxicity and others already in preclinical trials, have successfully tested drug effects in zebrafish (Rennekamp, 2015; Fang, 2016; Cui, 2016).

Zebrafish heart rate and action potential are more similar to those of humans, as observed by Arnaout and colleagues, 2007, than other animal models, also it presents highlighted genetics and regulatory networks similarities driving cell fate (Vacaru, 2014). Moreover, cardiac performance in adult zebrafish can be detected by new noninvasive methods. It can be assessed by advancing conventional echocardiography with speck-le-tracking analyses and changes in cardiac performance, and enables highly sensitive assessment of regional myocardial motion and deformation in high spatio-temporal resolution (Hein, 2015).

Then in vivo studies represent an essential step in drug development and toxicity study, and the zebrafish cardiotoxicity test has been reported very reliable, describing the potential toxicity of drugs to the human cardiovascular system (Zhang, 2003).

3. Detection of Doxorubicin Toxicity using different animal models

Doxorubicin (most used trade name, Adriamycin) is a potent anti-tumoral agent utilized as an important, broad anti-cancer drug to treat leukemia, lymphoma, breast cancer and small cell carcinoma of the lung (Johnson, 1996; Henderson, 2003; Peer, 2007; Woll, 2012). The ability of doxorubicin to kill rapidly dividing cells and, in turn, slow disease progression has been acknowledged for over 30 years (Ling, 1996, Tacar, 2013). Then, the introduction of this antineoplastic antibiotic is one of the major successes in oncology. Though, in spite of the

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pharmacological advantage associated with its use, doxorubicin presents toxicological effects on noncancerous cells as well, leading to cardiotoxicity and recalcitrant heart failure at high cumulative doses (Tacar, 2013). This damage can manifest itself as arrhythmia, arterial hypertension, thromboembolism, angina pectoris, myocardial infarction, or heart failure (Carbalho, 2014). For instance, a dose of anthracycline-doxorubicin of 500 mg/m^2 of body surface area causes cardiac complications in 4–36% of the treated patients (Schlitt, 2014). Thus, understanding the mechanism by why doxorubicin induces cardiac injury is crucial not only to avoid its cardiotoxic effect but also to improve the therapeutic use of doxorubicin.

Several reports highlight the cardiotoxicity of doxorubicin in different animal models (Table I), focusing on children safety, where its pharmacokinetics have been assessed, evaluating whether an age dependency in the clearance (CL) of doxorubicin exists, and concluding that the lower CL in younger population should be considered, together with pharmacodynamics. Those results are especially important in cardiotoxicity, being essential to select the future dose for a protocol (Völler, 2015). This issue has been addressed by Zhu et al., 2008, using juvenile mice, concluding that treatment with high cumulative doses of doxorubicin induced cardiomyocyte atrophy, myofiber disarray, low levels of cardiomyocyte apoptosis, and altered expression of structural and regulatory proteins, normalization from the treatment was observed after a 13-week recovery period. Mostly, the studies of doxorubicin-induced cardiotoxicity perform a single injection followed by evaluation within one week (Kim, 2008, Fischer, 2005, Delgado, 2004).

Table I. Existing studies on Doxorubicin cardiotoxicity

Model	DOX Study	reference
Juvenile mice	5 weeks DOX administration determines a decline in cardiac systolic function with cardiomyocytes atrophy, myofiber disarray, low levels of cardiomyocyte apoptosis, and altered expression of structural and regulatory proteins. 13-week recovery period bring back to normality.	Zhu W et al., 2008
mice	Meloxicam abrogates the cardio toxic effect of Doxorubicin in mice.	Hassan MH et al., 2014
mice	Resveratrol (RES) generates cardiovascular protective effects by a heme oxygenase-1(HO-1)-mediated mechanism.	Gu J et al., 2012
mice	Cannabidiol is able to improve DOX-induced cardiac dysfunction modulating mitochondrial function.	Hao et al., 2015
Rabbits and dogs	encapsulation in long-circulating pegylated liposomes reduce DOX cardiotoxicity	Peter K et al., 1999; Xin et al., 2011
dogs	Antioxidants (Lycium Barbarum polysaccharides and edaravone) possess cardioprotective effect against DOX-induced acute cardiotoxicity	Xin et al., 2011
zebrafish (embryo-larva)	High DOX doses had lethal effects, low DOX doses resulted in sub-lethal effects, malformations, and changes of heart rate.	Chang C et al., 2014
rats	DOX Genotoxicity evaluation. Enzyme-modified comet assay reported a significant induction of DNA damage in heart tissue	Mugimane G et al., 2014

Doxo has being study as well in combination with protective compounds documented by Simunek et al., 2009, who reported the effect of protective molecules and studied the underlying cardiotoxicity mechanism of doxorubicin. Simunek, Hassan et al., 2014 showed that two different doses of meloxicam present a potential cardio-protective effect, and by Gu et al., 2012 showed that a combination of resveratrol/doxorubicin in mice was able to generate cardiovascular protective effects by a heme oxygenase-1 (HO-1)-mediated mechanism.

Beside of the use of mice to evaluate cardiotoxical effect after drugs treatment, the zebrafish is presented as a costless vertebrate model with reduced complexity, and time, promising to be a powerful tool to evaluate cardiotoxicity. By, Chang et al., 2014, was evaluated, lethal and sub-lethal

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doses of doxorubicin in embryo-larva at different time points (4 and 120hpf, (hours post fertilization)), showing that higher doxorubicin doses had lethal effects, whereas lower concentrations resulted in sub-lethal effects and malformations, as well as changes in the heart rate (Chang, 2014). Chi et al., 2008, used the embryo transparency permitting detailed optical mapping to characterize the cardiac conduction system (CCS). Since heart rate measurement is quite easy in zebrafish, it makes it an attractive screening tool for assessing cardiovascular risk after treatment (Musso, 2014). Most importantly, zebrafish can survive in the absence of cardiac output and in the presence of major vascular defects for several days, unlike many of the larger animals (Rocke, 2009). Thence, all of those characteristics have made *Danio rerio* increasingly popular to test cardiotoxicity and cardiovascular developmental effects after drug administration as doxorubicin.

4. Antipsychotics toxicity testing

Antipsychotics are a class of medications primarily used to manage psychosis (including delusions, hallucinations, or disordered thought), particularly in schizophrenia and bipolar disorders, by alleviating such symptoms as hallucinations, both visual and auditory, and paranoid thoughts (Leon, 2010). However, the first generation of antipsychotics has usually been associated with elevated cardiovascular mortality due to QTc interval prolongation and may cause TdP. Many of them had to be withdrawn from the market, and starting from 2005, the ICH E14 guidance has recommended conducting a "thorough QT/QTc study" aimed at assessing whether the drug has an effect on QT interval (Lasser, 2002; Shah, 2006; Stockbridge, 2013). Normally, the early antipsychotic medications often have unpleasant side effects, leading researchers to continue their searches for better drugs, avoiding effects as severe ventricular arrhythmias and sudden cardiac death. The dysfunction of the cardioregulatory system may also be associated with functional and medication-related mechanisms rather than structural changes (Koponen, 2008).

Some studies have assessed the cardiotoxicity of certain antipsychotics in mammalian models (Table II). For instance, clozapine has been found to induce myocarditis in rats, which exhibited inflammatory response, myocyte vacuolar degradation, myofiber necrosis and interstitial fibrosis (Abdel-Wahab, 2014, Wang, 2008). Similarly, a cardiotoxic effect of clozapine in mice has been reported, detecting myocarditis, as well as inflammatory lesions after 7 or 14 days with 5, 10 or 25mg/kg dose daily treatment.

Table II. Existing studies about cardiotoxicity of Antipsychotic drugs.

Model	Antipsychotic Study	Reference
rats	Clozapine induces myocarditis, showing inflammatory respond, myocyte vacuolar degradation and myofiber necrosis	Abdel-Wahab BA et al., 2014
mice	7 or 14 days Clozapine daily treatment causes myocarditis as well as inflammatory lesions after	Wang JF et al., 2008
rats and mice	histological determination of cardiotoxicological effect of antipsychotic as aripiprazole, olanzapine, quetiapine, risperidone or ziprasidone	Wang JF et al., 2008
zebrafish larvae	cardiotoxicological effects of first generation antipsychotics (aripiprazole, clozapine, olanzapine, quetiapine, risperidone and ziprasidone)on heart rate, morphology and motility.	Lee SH et al., 2013

In spite of this evidence, there is a lack of systematic evidence of the cardiotoxicological effects of many antipsychotic drugs. On the other side, the cardiotoxicity of antipsychotics such as aripiprazole, olanzapine, quetiapine, risperidone and ziprasidone has not been investigated in rats, likely due to the high cost that such experiments would entail. As a result, most studies about the cadiotoxicity of these drugs in rats have relied on histological determination, which yields a poor understanding of their cardiotoxological effects (Wang, 2008; Dang, 2016). This underlines the need for alternative, more economical models for these experiments.

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The zebrafish emerges as a highly tractable model for toxicity studies of antipsychotics precisely for these reasons. Although mammalian toxicity studies remain the gold standard for risk assessment, the zebrafish has become a valid model due to the toxic responses that appear to be well conserved between mammalians and zebrafish (Barbazuk, 2000).

Lee et al., 2013, analyzed: aripiprazole, clozapine, olanzapine, quetiapine, risperidone and ziprasidone (first-generation antipsychotics) using the zebrafish larvae, where the heart rate, morphology and motility were measured. The authors concluded that the zebrafish model is an exceptional tool for cardiovascular risk assessment (Lee, 2013).

Thus, the use of the zebrafish as a model in these studies will facilitate more extensive, easy and comprehensive knowledge of those molecules cardiotoxicity, generating a deeper understanding of that process. Similarly, the zebrafish is also an attractive screening tool for cardiovascular risk assessment after treatment with atypical antipsychotic drugs, as it facilitates the evaluation of the heart beat rate (Christian Pylatiuk, 2014).

5. Animal model to evaluate genotoxicity

An important component in toxicology and drug development is to assess genotoxicity. This important issue has been evaluated through toxicological assays as, Ames test, comet, or *in vitro* and *in vivo* micronucleus assays, and in the past few years, *Danio rerio* has started to be considered as an *in vivo* alternative method to evaluate genotoxicity.

During years, rats have been extensively utilized to evaluate the genotoxicity of drugs through comet assay, micronucleus test and gene profiling techniques, reported by Manjanatha et al., 2014, where they found a significant induction of DNA damage in heart tissue after doxorubicin treatment using the enzyme-modified comet assay.

Nevertheles, in the last few years, start to appear zebrafish as a genotoxic tool. By Faßbender et al., 2013, using comet assay after methyl methanesulfonate treatment (an alkylating agent), it evaluated the presence of micronuclei after a treatment over two weeks, using adult zebrafish. Tissues as gonad, liver, or gild were analyzed. Concluding, that *Danio renio* is an efficient vertebrate model to study genotoxicity through comet assay and the micronucleus test. They argued that this model proved appropriate for the detection of genotoxicity in primary male and female gonad cells as well as using histological sections of the gonads from zebrafish, respectively.

Finally noteworthy, PAC2 zebrafish cell line has been used as well as an *in vitro* model in genotoxicity studies. A short-term (2 hours) exposure to a concentration range, reveals genotoxic pressure by genotoxic agents. As a note this cell line compared to another fish cell line (PLHC-1, trout hepatocytes), showed less sensitivity upon short-term exposure to the genotoxicants tested (Devaux, 1997; Šrut, 2011; Šrut, 2015). Thus, those reports bring to light zebrafish as in vivo and in vitro model to study genotoxicity.

6. Cardiotoxicity of Small Molecules: Screening

Cardiovascular toxicity is a major limiting factor in drug development and requires multiple cost-effective models to perform toxicological evaluation, however zebrafish are now a well-validated animal model to study treatment with small molecules, as well as to elucidate biological functions, and deciphering the mechanism of bioactive compounds (Chan, 2002). The model has emerged as a powerful system for small molecule screening and for novel biological and therapeutic discoveries. For instance through ISH (in Situ Hybridization), the expression of some target genes has been reported, the assay requires prior knowledge of the biologic process and depends on the selected molecular target, which should be critical for the developmental process (Jing, 2012).

In an attempt to evaluate toxicity of medicaments and other chemicals, new methodologies focusing on cardiomyocytes properties or computational models are under development (Cummins, 2016; Glinka, 2014). In spite of they are extremely useful, do not provide enough information about toxicity in the organism as an effect of the secondary metabolism derived from drugs, or the drug effect in other cellular lines present in the heart.

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Primary cardiomyocytes derived from human embryonic stem cells (hESC) (Holmgren, 2015) are used to evaluate cardiotoxicity, however the general consensus is that a reliable *in vivo* model is needed. Additionally some *in silico* approaches (computational techniques) can assess for instance, the flow for the action of a drug, reaching some results about Na+, K+, L-type Ca2+ channel or multiple membrane ion channels in cardiomyocytes. These computational models show up as cardiotoxicological method to evaluate the actions of drugs on cardiac electrical activities at cellular and tissue (Yuan, 2014).

Cardiac electrophysiology and modeling drug-channel, have documented many improvements in the last decades, although, the generation of a virtual heart model for drug safety assessment is still a mayor Challenge. Firstly, more studies are necessary to develop biophysically accurate models: the zebrafish could constitute a good overture for drug-channel interactions and cardiac electrophysiology. Although simpler than humans, zebrafish are also complex vertebrates that maintain similarly elaborate mechanisms to activate or relieve the effects of exogenous chemicals.

Thus, the close resemblance of the genetic cascade governing heart development in zebrafish to that of humans has propelled the zebrafish system as a cost-effective model to conduct pharmacological screens on developing embryos and larvae as well as to provide data to generate computational models to evaluate *in silico* drugs studies.

7. Modeling human diseases using the fish

Understanding syndrome with genetic bases has become an important topic in medicine, new treatments will appear to palliate those diseases, with a better understanding of the mechanisms that underlie genetic diseases. The zebrafish appears as a fast model to study de novo mutations and genetic diseases. Using genomic editing approaches as CRISPR/Cas9 (Hwang, 2013), or artificial site-specific nucleases such as zinc-finger nucleases (ZFNs), and transcription activator-like nucleases (TALENs), (Bedell, 2012; Doyon, 2008), genes can be inactivated in vivo, mimicking human phenotypes, and obtaining information about human diseases with genetic background.

For instance, a genetic syndrome called Dravet syndrome (DS), is linked to more than 300 *de novo* mutations present in a neuronal voltage-gated sodium channel (SCN). Dinday Matthew T, et al., 2015, reported to have screened a chemical library, constituted of around 1000 compounds. From this study, the authors identified four compounds with the ability to rescue the behavioral seizure component, and they reported that dimethadione, suppressed associated electrographic seizure activity. To reach such conclusions, the authors used a mutant zebrafish line called scn1lab DS. The relevance of this syndrome is, its association with a higher risk of sudden death in children, (Dravet, 2005; Ceulemans, 2012).

Similarly to the DS syndrome, other genetic modifications that lead to heart disorders associated with structural heart defects, can be found. For instance, the resembling human dilated cardiomyopathies (DCMs) (Stainier, 1996), that together with the silent heart, or the pickwick mutans, present poor heart contractility. DCMs is characterized by Ventricle or Atrium enlargement, or even DCMs can present the double phenotype. DCM syndrome has been studied with two particular mutant zebrafish lines: tnnt2 and laminin α-4 integrin linked kinase, both lines affected endothelial cells and cardiomyocytes, similarly to familial phenotype presented with DCM in human. (Knöll, 2007).

The description made by Mishra, 2013, shows the ability of zebrafish to simulate amyloid light-chain amyloidosis, a plasma cell disorder that causes rapidly progressive cardiomyopathy. Protein injection into blood torrent upon zebrafish embryos, led into a severe cardiomyopathy, showing a clear cardiac dysfunction, cell dead and edema pericardial.

Thus, the use of this model is uniquely positioned among vertebrates as a platform for small molecule screening: the zebrafish is used to identify novel drugs associated with molecular pathways, with the purpose to treat human diseases. The use of genetic manipulation in zebrafish is an efficient way to assess the roles of individual genes in disease processes. As such, it represents a route to the identification of novel drugs (Zon, 2005). In addition, zebrafish can be used to provide

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insight into the biological function of the many candidate genes being rapidly identified in human genome (Kettleborough, 2013).

8. Advantages of the Zebrafish Model with Respect to Cost and Time

As it has been described, toxicity testing of drugs in recent years has employed various animal models. Although mice, rats, rabbits and dogs are excellent models according to most standards, they present some serious limitations (Fig.1).

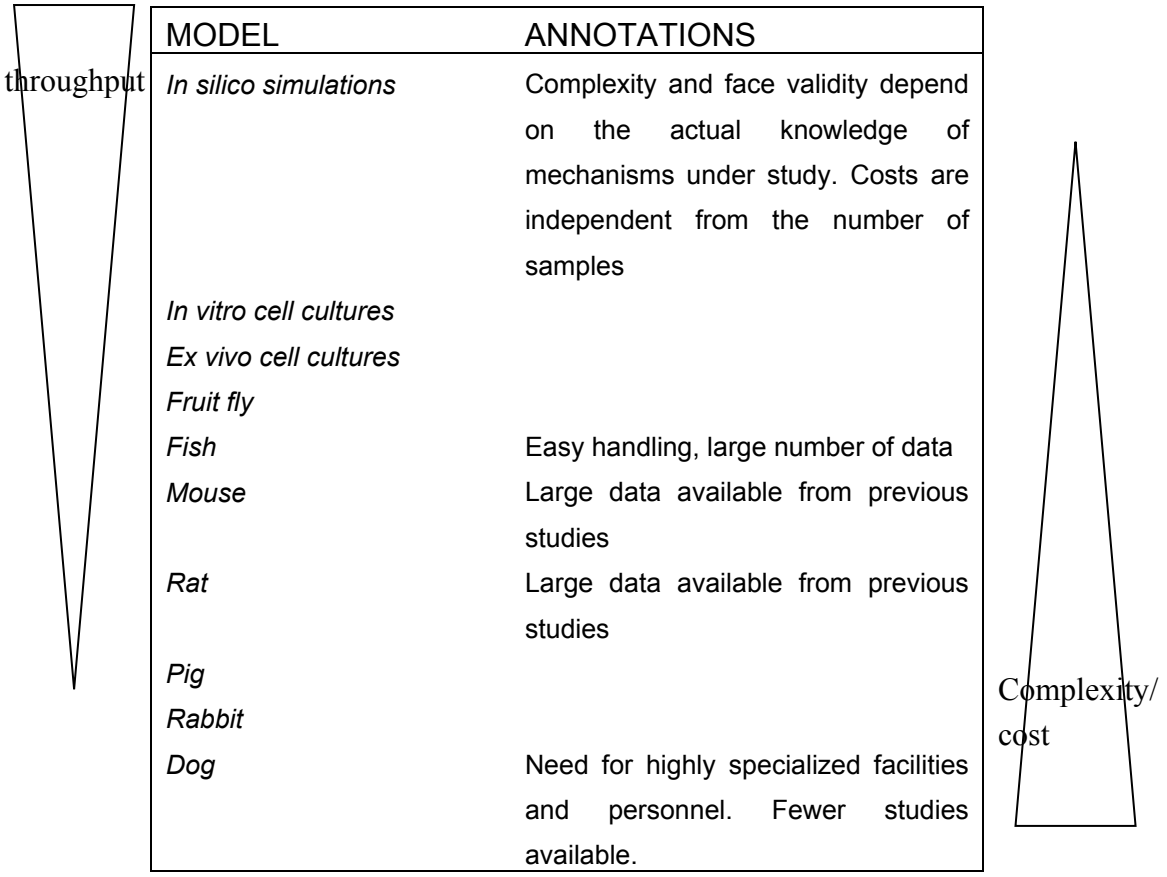

Fig.1. Representation of different biological system and animal model used to test cardiotoxicity drugs, classified in function of face validity, costs and throughput efficiency.

Experiments evaluating drug toxicity typically require large numbers of animals increasing the monetary cost of the experiments significantly as well as, those animals handling is often quite time-consuming. The small size of zebrafish renders them, ideal for experiments, being more easily handled and being associated with lower costs (Fig.1), and providing researchers and those concerned with animal welfare, with an alternative to work according to the Three Rs principles (refinement, reduction and replacement) (Schaeck M, 2013).

As a toxicology model, zebrafish have the potential to reveal the pathways of developmental toxicity due to their similarity with those of mammals. Zebrafish therefore, provide a sound basis for the risk assessment of drug administration in humans.

Thus, in many respects, the use of the zebrafish as a model for studies of cardio- behavior or genotoxicity would allow the researcher to overcome many of the challenges presented by using other animals models, including limitations on sample size and higher monetary and time costs.

9. Conclusion

Recent years have seen an increase in the number of studies evaluating the toxicity of drugs in various animal models. While larger animals such as mice, rats, rabbits and dogs are generally

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appropriate models to use, they present significant limitations, particularly with respect to cost, time, ethical concerns and sample size. On the other hand, *in vitro* tests used to assess biosafety lack the potency and the translational attributes of a whole animal.

Danio rerio is a good alternative for biosafety studies due to its small size, genetics background, higher breeding capabilities, and most importantly, due the similarities of its molecular pathways and physiology with that of humans. The emergence of zebrafish as a model for assessing cardio-neutotoxicity or genotoxicity of drugs is reflective of its advantages over other animal models in precisely these respects as well as its use following the principles of the 3Rs (Replacement, Reduction and Refinement). On the other hand, the ease of genome editing, using new mutagenesis techniques such as CRISPR/Cas9 (Hwang, 2013) in the fish, will make suitable future studies to pair human genetic mutations with their molecular functions.

In light of these advantages, we emphasize the zebrafish model as an excellent vertebrate toxicological model with potential to contribute to significantly improve drug development in toxicology.

In conclusion, the zebrafish presents a powerful *in vivo* preclinical model for assessing the adverse effects of a wide range of drugs. Its use, in conjunction with approaches based on those presented in this review, would contribute significantly to the literature and would facilitate the implementation of innovative, comprehensive, and cost-effective testing strategies.

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