Hepatotoxicity by Acetaminophen and Amiodarone in Zebrafish Embryos

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ABSTRACT:

Introduction: Liver is an essential organ for the detoxification of chemicals and play pivotal role in various biochemical and physiological processes hence liver toxicity is one of the major causes for the withdrawal of newly discovered molecules. Characteristics viz. high fecundity rate, transparent eggs, less maintenance cost, livelihood in E3 medium without external feeding make Zebrafish (Danio rerio) suitable vertebral model for the hepatotoxicity assay. **Methods:** On 3 dpf, embryos were exposed to various concentrations of Acetaminophen and Amiodarone in well plates. This procedure was followed up to day 5. Due to mortality observed in Amiodarone treated group G32 (15µM) treatment was not performed for. On 6 dpf, embryos were kept in lateral position in agar plates and photography was performed. Captured images were analysed through Image J software. **Results:** Mortalities were observed in Amiodarone treated groups on day 4 (7.1 % at 25 µM), on day 5 (78.5% and 68.2% at 25 µM and 15 µM) and day 6 (100%, 78.5% and 64.2% at 25 µM, 15 µM and 10 µM). Statistically significant decreased pixel intensity was observed at 1000 µM, 2000 µM and 5000 µM Acetaminophen concentrations and at 5 µM, 7 µM, 10 µM Amiodarone concentrations. **Conclusion:** Acetaminophen and Amiodarone have hepatotoxic potential in zebrafish embryos. Amiodarone induces embryo lethality at 10 µM. NOAEL for Amiodarone is 3 µM and for Acetaminophen is 500 µM.

Key words: Liver necrosis, Pixel intensity.

SUMMARY

Liver toxicity is a major cause of drug withdrawal in drug discovery. In this case, various advantages of zebra fish embryos provides handy model for the early assessment of the liver toxicity. Minute quantity of the discovered molecule is sufficient to check its fate towards liver toxicity. This paper provides validated method to assess the liver toxicity in zebrafish embryos and that can be used to select doses of Acetaminophen and Amiodarone for further zebrafish toxicity studies at different age stages. Results of this study support the use of zebrafish embryos to predict hepatotoxicity in vertebrates as the results obtained are in line with the proven hepatotoxic effects of both the drugs in humans.

PICTORIAL ABSTRACT
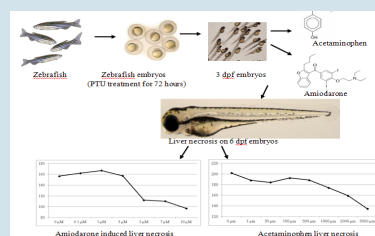
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INTRODUCTION

Zebrafish achieving more attention of the researcher recently particularly the scientist involved in the pharmaceutical background for the evaluation novel chemical entity for its efficacy and safety. It is well suited vertebral model because they have high degree of genetic conservation compared to human.[1,2] The size, embryonic and larval development gives additional advantages over other *in vitro* and mammalian model. Zebrafish embryos are transparent so it is very easy to observe organ development. The size of embryos is very small which enable to put the larvae in a petriplates and further in 12/24/96 well plates. This makes easy to change the medium and chemical treatment. Zebrafish embryogenesis completes within 72 hour post fertilisation. At this time liver is perfused with blood and fully functional. Development of physiologically functional liver is rapid compared with other vertebral model. In addition, the structure and function of a healthy adult zebrafish liver is generally the same as in mammals.[1,2] Thus, zebrafish may be promising non-rodent alternative model in toxicology which has an advantage over the traditionally used models.[3,4]

Acetaminophen has been widely used for > 50 years in the treatment of pain and fever and provides for the safe and effective relief of these symptoms. In a small minority of patients, however, acetaminophen is responsible for life-threatening liver injury and accounts for up to 50% of all adult cases of acute liver failure in the United States.[4]

Similar to its pharmacological action, Amiodarone adverse reaction profile is complex, ranging from thyroidal to pulmonary ocular and/or liver toxicity.[5]

To study hepatotoxicity at the early life stage of zebrafish i.e. embryos stage, we used Acetaminophen and Amiodarone. The objective of this research was to check the concentration wise hepatotoxic effects of Acetaminophen and Amiodarone in zebrafish embryos, to predict No-Observed Adverse Effect Level (NOAEL) and to validate method for assessing hepatotoxicity in zebrafish embryos.

Figure 1: Zebrafish embryo from control group.

MATERIALS AND METHOD

Materials

Chemcials

Chemical Name (1):	Acetaminophen
Lot N°:	SLBH0185V
Analysed Purity:	>99 %
Manufactured by:	Sigma-Aldrich
Chemical Name (2):	Amiodarone
Lot N°:	SLBF5607V
Analysed Purity:	> 99%
Manufactured by:	Sigma-Aldrich

Instruments and Equipment

Micropipette of Eppendorf and Brand, Germany, 12/24 well plates of Corning, Costar USA, Stereoscope of Stereodiscovery V.12, Carl Zeiss Germany, Incubator of Remi Sales and Engineering India, Zebrafish Housing System of Aquaneering USA and Centrifuge tubes of Corning, USA

Method

Couples of F1 Generation zebrafish were kept for crossing and eggs were collected. Normal eggs were allotted for the treatment. The day of egg collection was considered as day 1. Embryos were kept in petri plates containing E3 medium and kept at 28.5°C temperature. On day 1, the embryos were treated with 0.003% PTU in E3 medium which was continued for next 3 days.

On 3 dpf, embryos were transferred in well plate and the stock concentrations for Acetaminophen and Amiodarone were prepared by mixing the test item in E3 medium and DMSO based on the solubility. Aliquots of stock concentrations were taken to prepare final concentrations of 1 µM, 50 µM, 100 µM, 500 µM, 1000 µM, 2000 µM and 5000 µM for Acetaminophen and 0.1 µM, 1 µM, 3 µM, 5 µM, 7 µM, 10 µM, 15 µM and 25 µM for Amiodarone.

Final test concentrations were prepared in 0.003% PTU in E3 medium and final test concentrations were renewed on next day. This procedure was followed up to day 5. Due to mortality observed at 15 µM in Amiodarone treated group, further treatment was not performed.

Figure 2: Zebrafish embryo from treatment group.

RESULTS

All the embryos for both the treatment groups were observed daily up to 6 dpf (till photography) and embryos were found normal for Acetaminophen treated groups.

7.1 % mortality was observed at 25 µM on day 4 in Amiodarone treated group.

78.5% and 68.2% mortality was observed at 25 µM and 15 µM groups respectively on day 5 in Amiodarone treated groups. 100%, 78.5% and 64.2% mortality was observed at 25 µM, 15 µM and 10 µM groups respectively on day 6 in Amiodarone treated groups.

On day 6, embryos were anaesthetized with Tricaine and observed for liver necrosis (darkness). Embryos were kept in lateral position in Agarose plate and photography was performed on Carl Zeiss Stereodiscovery V12 microscope. After taking all the photographs, the images were analyzed by Image J software for its pixel intensity. Measured pixel intensities were then analysed by Bartlett's test, Anova Test and Dunnett's t-test and observed for the significance.

Statistically significant decreased pixel intensity was observed at 1000 µM, 2000 µM and 5000 µM Acetaminophen concentrations. Statistically significant decreased pixel intensity was observed at 5 µM, 7 µM, 10 µM Amiodarone concentrations.
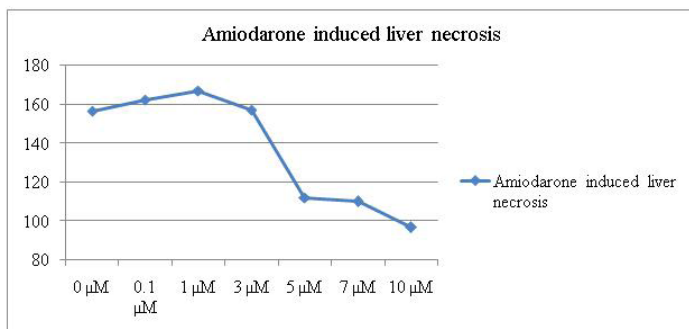

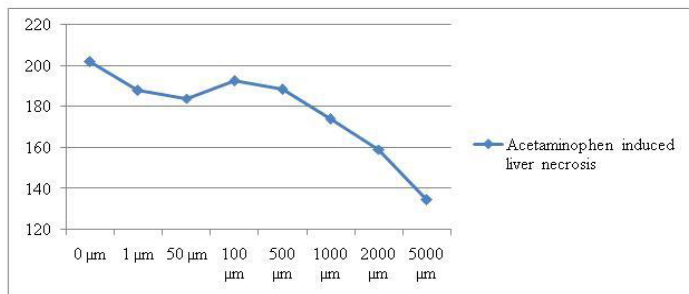**Figure 3:** Concentration of acetaminophen (µM) Vs. pixel intensity.

Figure 4: Concentration of amiodarone (µM) Vs. pixel intensity.

DISCUSSION

Drug induced liver injury accounts for approximately 11-13% of acute liver failure cases in the Unites States.[6] Therefore, it's a keen requirement to study the newly discovered molecule with respect to its hepatotoxicity. Drug discovery has many stages from identification of molecule to identification of target where molecule actually acts either advantageous or disadvantageous in the terms of drug receptor interaction. Applicability of the zebrafish models is quite influencing due to ready availability, easy maintenance so many other usefulness. The zebrafish model is best suited with the existing approaches to predict hepatotoxicity.[6]

Drug induced liver injury is a major cause of concern for its withdrawal from its early stages of drug development to post marketing surveillance i.e. phase IV of the clinical trial. The development cost of the pharmaceuticals is very high and failure of any molecule extends the process. In this situation, tier approach for estimating the molecules in zebrafish reduces the cost of assessing the toxicity.[2]

Drug discovery has variety of physiological, biochemical and cellular assays to evaluate biodiversity at early phase. Tiny model of zebrafish and its sets of assay like hepatotoxicity, cardiotoxicity, teratotoxicity, ototoxicity and inhibition of angiogenesis provide useful information of new drug candidate. Zebrafish is an emerging vertebrate model for drug discovery that permits whole animal drug screens with excellent throughput, combined with ease of use and low cost.[7] Zebrafish embryos are relatively small during early life stages so minute quantity of the drug is required to check the activity of the molecule which helps researchers to develop the molecule at the laboratory before large scale production at industry level where they readily check the potency of the compound.

Though significant risk of hepatotoxicity by the developed compound never be judge unless phase I trials, early hepatotoxicity in zebrafish gives approximation to decide the safer dose in higher primates and finally in humans. Transcript profiling by microarray and quantitative polymerase chain reactions have revealed that 94 zebrafish CYP genes, most of which are direct orthologs of human CYPs, are expressed in embryos during various time courses associated with development has further investigated the expression of several xenobiotic metabolizing genes similar to human (e.g., CYP1A, CYP2B6, CYP3A5, and UGT1A1) and functional metabolizing capacity using several classic CYP substrates over a time course of early development (i.e., from 24 to 96 hpf).[2, 8, 9]

Zebrafish has more relevance with a human which predicts the actual effects of chemical which deal with other mammalian models in rest of the assays. But many CYP enzymes behave differently in each mammalian model which acquires the usage of various genetic assessment techniques.

CONCLUSION

Acetaminophen and Amiodarone have hepatotoxic potential in zebrafish embryos. Amiodarone induces embryo lethality at 10 µM. NOAEL for Amiodarone is 3 µM and for Acetaminophen is 500 µM. Results of this study support the use of zebrafish embryos to predict hepatotoxicity in vertebrates as the results obtained are in line with the hepatotoxic effects of both the drugs in humans.

SUMMARY

- Liver toxicity is a major cause of drug withdrawal in drug discovery. In this case, various advantages of zebra fish embryos provides handy model for the early assessment of the liver toxicity. Minute quantity of the discovered molecule is sufficient to check its fate towards liver toxicity. This paper provides validated method to assess the liver toxicity in zebrafish embryos and that can be used to select doses of Acetaminophen and Amiodarone for further zebrafish toxicity studies at different age stages. Results of this study support the use of zebrafish embryos to predict hepatotoxicity in vertebrates as the results obtained are in line with the proven hepatotoxic effects of both the drugs in humans.

ABBREVIATIONS USED

There are no any typical abbreviations used in manuscripts.

ABOUT AUTHOR

Mehul Pandya pursued M.Pharm. in pharmacology from Gujarat Technological University. Presently working at Jai Research Foundation as a research officer and study director for varieties of toxicity studies which are to be conducted as per national and international regulatory guidelines.

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