HEPATOTOXICITY BY ACETAMINOPHEN AND AMIODARONE IN ZEBRAFISH EMBRYOS

ABSTRACT
Liver is an essential organ for the detoxification of ingested 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. Peculiar 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. On 3 day post fertilisation (dpf), embryos were treated with various concentrations of Acetaminophen and Amiodarone for 72 hr. Embryos from both treatment and control groups were observed daily for mortality and other abnormalities. No mortality was observed in Acetaminophen treated groups. At 25 µM, 15 µM and 10 µM Amiodarone concentration, 100%, 78.5% and 64.2% mortality was observed, respectively and captured images of embryos were analysed to evaluate liver necrosis through Image J software on 6 dpf. Significantly decreased mean pixel intensity was observed at 1000 µM, 2000 µM and 5000 µM Acetaminophen concentrations compared to control group. Significantly decreased mean pixel intensity was observed at 5 µM, 7 µM and 10 µM Amiodarone concentrations compared to control group. Based on the results, it is concluded that, NOAEL for Amiodarone and Acetaminophen is 3 µM and 500 µM respectively. Both molecules are potentially hepatotoxic for zebrafish embryos and the 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.

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. 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. Thus, zebrafish may be promising non-rodent alternative model in toxicology which has an advantage over the traditionally used models.

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

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.

MATERIALS AND METHOD

Materials

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<thead>
<tr>
<th>Chemical Name (1)</th>
<th>Analysed Purity</th>
<th>Manufactured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>&gt;99 %</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Acetaminophen</td>
<td>&gt;99 %</td>
<td>Sigma-Aldrich</td>
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Chemicals

- Chemical Name (1) : Acetaminophen
- 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 Stereidoscopy 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 0. 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 0.1 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.

RESULTS
Rall 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 Stereoscopy V.12 microscope. After taking all the photographs, the images were analyzed by Image J software for its pixel intensity. Measured pixel intensities were then analyzed 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.
CONCLUSION
Based on the results, it can be concluded that Acetaminophen and Amiodarone have hepatotoxic potential in zebrafish embryos. Amiodarone induces embryo lethality at 10 µM. Lower concentrations induce hepatotoxicity. NOAEL for Amiodarone is 3 µM and for Acetaminophen is 500 µM. No developmental alterations were observed in any of the treatment groups.

REFERENCES

- Acetaminophen safety and hepatotoxicity – where do we go from here? July 2007, Vol. 6, No. 4 , Pages 341-355 (doi:10.1517/14740338.6.4.341) Patrik J Amar, and Eugene R Schiff University of Miami Miller School of Medicine, Division of Hepatology, 1500 NW 12 Avenue, Suite 1101 East Tower, Miami, Florida 33136, USA
- Olaia Holgado, María Diez, Carles Callol-Massot, M. Luz Martinez-Chantar, Ainhoa Letamendia Prediction of hepatotoxicity in zebrafish, BIOBIDE S.L Donostia-San Sebastián, Spain