A pilot study on the effects of mechanical dechorionation on developmental toxicity in zebrafish embryos

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Abstract

Prior to hatching, the zebrafish embryo is surrounded by a chorion. This acellular membrane predominately consists of glycoproteins and is thought to be freely permeable to water and small molecules. However, when studying possible embryotoxicity, the intact chorion may influence the accessibility of test compounds to the embryo and results may depend on their physicochemical properties. Thus, there is a possibility of generating false negative results in developmental toxicity studies due to negligible permeation of the compound across the chorion. In the present pilot study, four compounds with a wide range of log P values (cytosine arabinoside, hydroxyurea, dimethadione and valproic acid; log P values: -2.46, -1.8, 0.18 and 2.75, respectively) were tested for developmental toxicity in zebrafish embryos of the WIK strain with the chorion intact or dechorionated. Since this was a pilot study and a relatively low number of embryos (5-16) per concentration were used, it was difficult to draw firm conclusions with respect to the influence of the chorion on the observed embryotoxicity elicited by the compounds. Presently, it was demonstrated that the mechanical process of dechorionation, with fine forceps at 4 hours post fertilization (hpf), appeared to be detrimental to the embryos, and in some instances caused a high frequency of mortalities and toxic effects in the embryos. The results also indicated that hydrophilic compounds (cytosine arabinoside and hydroxyurea) might be toxic at lower concentrations in dechorionated embryos than in embryos with an intact chorion. For dimethadione, with a higher log P value, the presence of the chorion did not appear to significantly influence the embryotoxicity of this compound. Dimethadione caused a concentration-dependent decrease in pH, which may cause adverse effects in the embryos. However, when the pH was adjusted no lethality but similar abnormalities were observed. It was concluded that mechanical dechorionation is difficult and involves a high risk of damaging the embryos. Based on this pilot study, we recommend that the physicochemical properties of both hydrophilic and lipophilic chemicals, and test solution parameters (pH and osmolarity) also need to be considered in the experimental design and the evaluation of results from zebrafish embryos developmental toxicity studies.

Methods

- WIK strain embryos were mechanically dechorionated at 3 – 4 hpf, using fine forceps.
- At 4 hpf dechorionated and chorionated embryos were transferred with 500 µl of embryo medium into agarose coated 12 well plates which contained the test compound.
- For each compound the same clutch of embryos was used for both dechorionated and chorionated experiments.
- Exposure:

- In each experiment, embryos were exposed to five concentrations of each test compound (two wells per conc) plus medium as controls (one well), with 5-8 embryos per well. The compounds were dissolved in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 5 mM HEPES, pH 7.2) and the test solution was refreshed at 27 hpf. At 48 hpf embryos were washed and incubated in embryo medium until 7dpf, with replenishment of medium every day. Temperature, conductivity, pH and dissolved oxygen was monitored during the study.
- The embryos were scored at 25 hpf, 48 hpf, 72 hpf, and at 7 dpf when the larvae also were stained with alcian green for cartilage. Dead embryos were discarded at every replenishment of solution. The criteria for lethality was at 25 hpf: coagulated eggs; and for the other occasions: no heart beats in combination with no circulation.
- Scored abnormalities at 7 dpf were abnormal dorsal ventral swim position, abnormal gut, abnormal cartilage, abnormal startle response, abnormal body shape. The reported incidence of abnormalities is the number of embryos displaying any abnormalities divided by the total no of viable embryos at 7 dpf.
- pH experiment:
  The pH of the dimethadione-test solution was adjusted to 7.2 using NaOH, and the pH of the embryo medium was adjusted to 5 using HCl.

Aim

To study the impact of dechorionation of zebrafish embryos on developmental toxicity using four test compounds with varying lipid solubility. In addition, the effect of pH in the test solution was investigated.

Results

A. Effects of dechorionation

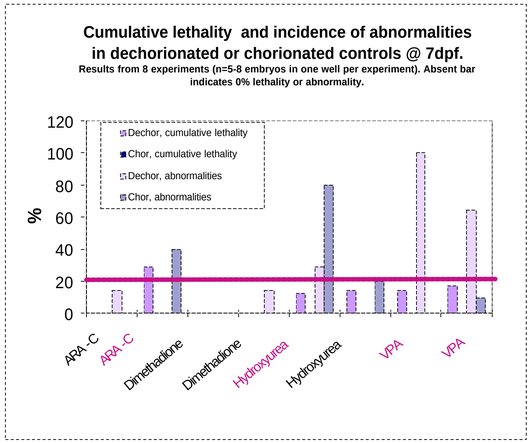
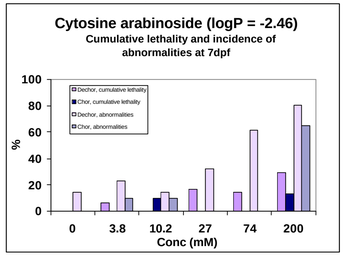
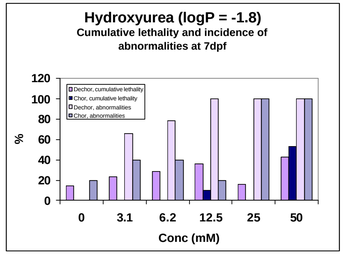


Fig. 1 Results demonstrate that mechanical dechorionation with fine forceps is detrimental to embryos in many instances. Experiments with cumulative lethality and/or incidence of abnormalities at 7dpf above the threshold (20%) were not included in further evaluation. This is the reason why VPA results are excluded from presentation.

B. Effects of lipid solubility (logP)





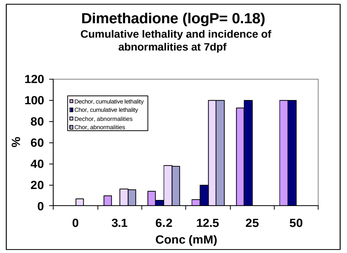


Fig 2. These preliminary data indicate that for cytosine arabinoside and hydroxyurea (highly hydrophilic compounds) embryotoxicity was seen at lower concentration in dechorionated embryos compared to chorionated embryos. For dimethadione no significant difference was observed.

C. Effects of pH

For dimethadione a conc-dependent decrease of test solution pH was observed (6 at 6.2 mM to 5 at 50 mM). Recommended pH for embryos is 7.2. It is thus possible that the high lethality and abnormalities (fig 2) observed in embryos exposed to this compound may have been due to the low pH rather than toxic effects of the compound.

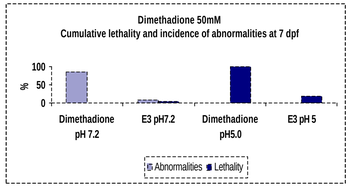


Fig 3. Adjusting the pH to 7.2 of the dimethadione test solution resulted in no lethality, but the majority of the embryos were still abnormal (see fig 4). Incubation of embryos in embryo medium "E3 pH 5", without dimethadione, resulted only in 20% lethality. However, as the pH of "E3 pH5" had returned to 7.2 at replenishment, the effect could be underestimated. The results indicate that dimethadione in itself causes specific abnormalities, while at a low pH with the same concentration the effect is lethal.

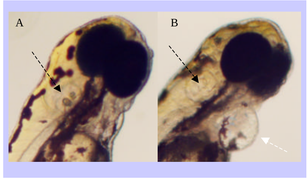


Fig 4. A, control embryo (72 hpf), black arrow point to the two otoliths. B, embryos treated with 12.5 mM dimethadione without otoliths (black arrow). This embryo has also pericardial swelling (white arrow). The same pattern was also seen after treatment with 50 mM dimethadione pH 7.2.

Conclusions

- Mechanical dechorionation was detrimental to the embryos.
- For the compounds with low log P values (hydrophilic compounds), it seems as toxicity occurs at lower concentrations in dechorionated embryos than in chorionated embryos. This might indicate that the chorion limits the transport of these substances.
- pH of the incubation medium can be affected by the test compound and this possibility should be considered in developmental toxicity studies in zebrafish embryos.

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