A quantitative and mechanism-specific analysis approach for the fish embryo toxicity test

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1. Introduction

To date, the application of the zebrafish embryo toxicity test (zFET) has focused on the assessment of acute toxicity, where only lethal morphological effects are accounted for. For applications beyond the acute toxicity, the test requires more refined and preferably quantifiable toxicological endpoints. Valuable tools in this context are fluorescent markers, which can either be expressed *in-vivo* or used for antibody-coupled labelling. The fluorescent signal facilitates the visualisation of specific cells or morphological structures and allows for quantitative measurements. Moreover, underlying toxicological mechanisms may be elucidated or the detection of adverse effects enhanced, what, in turn, increases the sensitivity and specificity of the conventional zFET [1]. This project explores the benefits of fluorescent marker applications in the zFET for studying chemical toxicity and effects on the vascular, myotomal, and neuronal development. Corresponding methods to apply and analyse the fluorescent read-outs in a quantifable and standardised manner are developed and integrated in the zFET procedures. By the example of genistein exposure, we could demonstrate that the integration of different fluorescent signal based methods allowed a more specific identification of toxicity mechanisms and reduced the error rate of the non-lethal effect assessment.

2. Materials and methods

Zebrafish embryos of two transgenic lines, *Tg*(*fli1*:EGFP)^{y1} and *Tg*(*gfap*:GFP) and the wild type strain were exposed to genistein concentrations of 2 – 4 mg/L for 48 h. The transgenic line *Tg*(*fli1*:EGFP) expresses enhanced GFP in the vasculature and thus enables the visualisation of vascular defects in live embryos [2]. *Tg*(*gfap*:GFP) expresses GFP together with the glial fibrillary acidic protein (gfap) gene in the notochord and facilitates the identification of potential neurotoxic effects [3].

The embryos were assessed microscopically for lethal and non-lethal effects after 24 and 48 h of exposure following available guidance documents for the zebrafish embryo toxicity test (zFET) [4] [5]. Additionally, the specific GFP-expression of the transgenic lines was qualitatively and quantitatively analysed using fluorescence microscopy and image analysis with ImageJ.

Whole-mount immunofluorescence was applied to visualise myotomal defects and *in-vivo* staining for assessing chemical induced apoptosis.

3. Results and discussion

3.1. Morphological effects

Genistein-exposed embryos exhibited pericadial edema, reduced blood circulation and notochord deformations, which became significant at concentrations of 4 mg/L after 24 h and at 2.38 mg/L after 48 h of exposure. The calculated EC50 value was 2.44 mg/L.

Using the fluorescence assessment methods, the zFET showed additionally impaired vasculogenesis, glial development and myotome morphology deficits (data not shown) and induced apoptosis in zebrafish embryos after genistein treatment (Figure 1). However, within the fluorescence assessment we could observe already significant effects with 2 mg/L genistein. Figure 1 depicts the concentration-dependent decrease of the relative blood vessel length and the fluorescent signal intensity in the notochord as well as an increase in the apopototic cell density in the embryos.

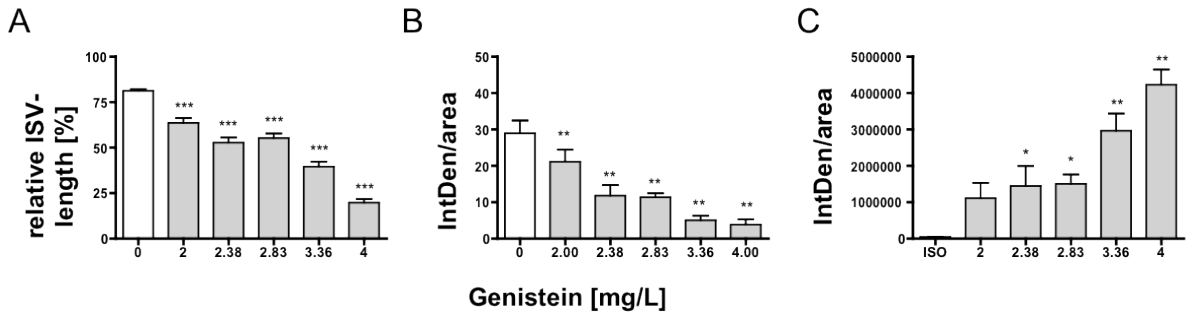

Figure 1: Results of genistein exposure of 48 h. *Measurement of the relative intersegmental blood vessel (ISV) length (%) of the Tg(fli1:EGFP) embryos for vasotoxicity (A), of fluorescence intensity of the Tg(gfap:GFP) embryos (IntDen/area) for neurotoxicity (B) and of fluorescence intensity of the in-vivo staining for cytotoxicity (IntDen/area) (C) assessment.*

Genistein induced apoptosis in specific cells, which were primarily located in the notochord region, as shown by double staining methods. This suggested a direct link between the apoptotic cells and the decrease in fluorescence in the notochord of the *Tg*(*gfap*:GFP) embryos.

4. Conclusions

The fluorescence signal of transgenic lines and antibody staining enabled the detection of vascular, myotomal and neuronal malformations in the embryos, which were not visible in bright field assessments. The fluorescent signal-based methods allow a sensitive and quantitative effect assessment, which will broaden the scope and minimize the data variability of the zFET. The combination of fluorescent and non-fluorescent endpoints facilitates an integrated effect analysis for a better mechanistic understanding of toxic action in the zFET.

5. References

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