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Research article

A rapid assessment for predicting drug-induced hepatotoxicity using zebrafish

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ABSTRACT

Introduction: Zebrafish have been used as a model to access drug-induced hepatotoxicity. However, individual differences occur in the liver development of zebrafish.
Methods: We used a transgenic line of zebrafish that expressed enhanced green fluorescent protein (EGFP) in the liver and then used a calculation of the liver index area, a potentially new endpoint of hepatotoxicity, to evaluate drug-induced liver injury. To further validate the reliability of the liver area index as a quick evaluation of zebrafish liver function damage, the liver area index level was correlated with hepatic transaminase activities using the Pearson correlation coefficient and confirmed by histopathology.
Results: Zebrafish larvae treated with high doses of the known mammalian hepatotoxic drugs carbaryl, isoniazide, and pyrazinamide showed significantly decreased liver area index levels, which are suggestive of liver injury and correspond with the higher alanine transaminase (ALT) and aspartate transaminase (AST) activities and histological liver alterations. The results showed a significant negative correlation between the degree of liver injury and the liver area index level.
Discussion: Our data support the use of the liver area index as a reliable and comparable indicator to screen hepatotoxic agents using the zebrafish model.

1. Introduction

Drug toxicity is a major toxicological challenge in the development of the pharmaceutical industry, and drug-induced liver injury is the most common adverse drug effect (Bissell, Gores, Laskin, & Hoofnagle, 2001). With the development of new drugs, the incidence of drug-induced liver injury has also increased accordingly. Many drugs, herbs, and other health care products also have the potential to cause liver damage (Frenzel & Teschke, 2016; Teo et al., 2016). Drug-induced liver toxicity is one of the main reasons for the failure of drug research or for withdrawal from the market of approved drugs, such as troglitazone (Norris, Paredes, & Lewis, 2008; Fontana, 2014; Gomez-Lechon, Tolosa, & Donato, 2015).

Although the early liver toxicity screening of candidate compounds and the potential hepatotoxic reevaluation of drugs has caught the attention of many researchers, a highly predictive animal model with simple analysis and a precise evaluation index is still relatively scarce (Sarges, Steinberg, & Lewis, 2016). Toxicity assessments of a drug prior to clinical trials are usually accomplished with laboratory rodent studies and liver cells in vitro experiments (Eun et al., 2015; Sison-Young et al., 2016). However, these detection methods have certain limitations. Cell experiments cannot accurately reflect the activity of drugs in the in vivo microenvironment. Mammalian models have the advantage that the results are reliable and comprehensive, but they require large amounts of drugs, extensive funds, and they are time-consuming and are thus unsuitable for high-throughput screening. Therefore, the traditional approaches for identifying hepatotoxicants are insufficient and there is a requirement for new models and technologies need to be developed.

Recently, zebrafish has been a useful vertebrate model for toxicology, drug-screening, and human disease studies (Gamse & Gorelick, 2016). The zebrafish copy of the human disease is not only highly similar but is also reliable, controllable, and repeatable (McGrath & Li, 2008). Zebrafish larvae are small and transparent during early life stages, and the endpoint of hepatotoxicity can be monitored via morphological changes that are visualized with transmitted light without

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the need for dissection (Johnson & Zon, 1999; Hill, Mesens, Steemans, Xu, & Aleo, 2012; Lee et al., 2015). Therefore, larval zebrafish studies can be performed with single milligrams of compound in microtiter plates, essentially allowing high-content in vivo information to be gathered in an in vitro format. The early embryonic stages of hepatogenesis are similar to that of mice (Field, Ober, Roeser, & Stainier, 2003), and the structure and function of the zebrafish liver is generally the same as in mammals (Hinton & Couch, 1998). Zebrafish have been used as a universal preclinical model organism for drug-hepatotoxicity screening in vivo (Vliegenthart, Tucker, Del Pozo, & Dear, 2014; Mesens et al., 2015). For example, North et al. developed a zebrafish model for acetaminophen liver toxicity and identified therapeutics that worked cooperatively with *N*-acetylcysteine (North et al., 2010). Zhang et al. tested different hepatotoxins using a transgenic zebrafish line with liver-specific DsRed expression (Zhang et al., 2014b).

In this study, we used a transgenic line of zebrafish that expressed enhanced green fluorescent protein (EGFP) in the liver and used liver area index, a new endpoint of hepatotoxicity, to evaluate drug-induced liver injury. Our data support the use of the liver area index as a reliable and comparable indicator to screen hepatotoxic agents using zebrafish.

2. Materials and methods

2.1. Chemicals

Carbaryl (CAS No. 63-25-2), purchased from the Shanghai pesticide research institute (China), was dissolved in dimethyl sulphoxide (DMSO) to make a 20 mM stock solution. Pyrazinamide (CAS No. 98-96-4) and isoniazide (CAS No. 54-85-3), purchased from Sigma (St. Louis, MO, USA), were dissolved in double-distilled water (ddH_2O) to make 20 mM stock solutions. The stock solutions were stored at −20 °C in darkness until use. Serial dilutions were made with fish water before experiments. The fish water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$ and 0.16 mM $MgSO_4$ per 100 mL distilled water, pH 6.9– 7.2, conductivity 480– 510 μS/cm) was prepared daily and was filtered before usage by zebrafish water cycle equipment (Beijing ESEN Science & Technology Development Co., Ltd, China) (Yang et al., 2011; He et al., 2012). Test solutions of carbaryl were prepared by dilution of the stock solution with the final concentration not above 0.5% DMSO.

2.2. Zebrafish

Tg (L-FABP: EGFP) transgenic zebrafish was obtained from Zebrafish Drug Screening Platform of Shandong Academy of Sciences, and the details about the generation of the line were described previously (Her, Chiang, Chen, & Wu, 2003). Healthy 3-month-old zebrafish were maintained at 28 °C with a 14:10 light:dark cycle and fed twice daily with brine shrimp. The zebrafish embryos were obtained from spawning adults in groups of 2 males and 1 female in a translucent plastic tank within 30 min after the onset of light in the morning. The embryos between fertilization and 72hpf were cleaned and then maintained in 2 L tanks (300 embryos per tank) containing 1 L of fresh fish water at 28 °C. The fish water was renewed daily and dead individuals were removed immediately.

2.3. Drug treatment

Healthy and normally developing larval zebrafish expressed EGFP in the liver were selected at 72 hpf by preliminary screening for fluorescence and were distributed into six-well cell culture plates (10 larvae per well in 5 mL of solution). A maximum tolerated dose exposure was conducted before the dosing of large groups of larvae (data not shown). The sublethal concentrations of the test compounds ranged from no or small effect on the liver to clear the toxic effect on the liver. Zebrafish were exposed to different concentrations of carbaryl (5, 10, 15, and 20 μM), isoniazide (4, 8, and 16 mM) and pyrazinamide (1, 2.5, and 5 mM) for a period of 72 h at 28 °C. Zebrafish were treated with 0.5% DMSO or fish water was used as vehicle control. The exposure solution was renewed every 24 h to keep the appropriate concentration of drug and water quality. Zebrafish were not fed during the assay. Because zebrafish larvae receive nourishment from their yolk sac, no feeding is thus required for the first 7 dpf (He et al., 2012). The study was carried out in triplicate. After treatment, zebrafish were subject to the liver toxicity testing.

2.4. Liver area index

After anesthetizing with 0.16% Tricaine (pH 6.9– 7.2), larvae were fixed on the slide in a lateral view using 3% methyl cellulose. The liver fluorescence in the larvae was observed under a green fluorescent microscope with GFP filter (470 nm wave length) and photographed using a digital camera (Olympus SZX16; Tokyo, Japan) with sufficient exposure time to show the whole liver region for liver area measurement at ×2.5 magnification. The whole larval lateral view was observed and photographed with a bright field microscope using a stereomicroscope (Olympus SZX16; Tokyo, Japan) with sufficient exposure time to show the whole larvae lateral region for larvae lateral area measurement at ×2.5 magnification (Fig. 1). The fluorescence and bright field images were taken on same scope and at similar time for each larva (10 per treatment and three replicates). For each larva, the fluorescence image was measured for liver area, and the bright field image was measured for larvae lateral area using Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA) which is a 2D analysis program. Using the measurement mode and the polygon tool, the region of interest was selected and the parameter of area was measured by Image Pro Plus software. The liver area index was calculated based on the formula:

$$\text{Liver area index} = \text{liver area}/\text{larvae lateral area} \times 100\%.$$

2.5. Histopathology evaluations of the zebrafish liver

For histopathological examination, larval zebrafish were fixed in 4% paraformaldehyde, gradually dehydrated in ethanol and embedded into paraffin (Hill, Howard, & Cossins, 2002). Embedded zebrafish larvae were longitudinally sectioned at 5 μm and stained with hematoxylin and eosin (H&E) (Sabaliauskas et al., 2006). For each larva, about thirty slide sections were obtained. Ten larvae were used for each group. The slides were photographed at ×40 magnification using a histological microscope (Bio Imaging Navigator FSX100, Olympus, Japan). Pathological diagnosis was conducted blind to prevent any bias on the slide sections selected and assessed.

2.6. Transaminase analysis

For the hepatic transaminase activity analysis, 150 larvae per sample were collected and homogenized in cold saline (Shandong Hualu Pharmaceutical Co., Ltd, China) after the hepatotoxic drug treatments. These larvae were separate groups post dosing not those post image analysis. 50 mg of larvae was homogenized in 450 μL cold saline. The homogenates were centrifuged at 2500 rpm (615 rcf or 8.8 rad) for 10 min, and the supernatants assayed for alanine transaminase (ALT) or aspartate transaminase (AST) activities using the spectrophotometric diagnostic kits according to the manufacturer's protocols (Nanjing Jiancheng Biotechnology Institute, China) as reported previously (Zhang de et al., 2016).

2.7. Statistical analysis

The coefficient of variation (CV) was calculated as the standard deviation divided by the mean (STDEV/mean) for each data point. All data