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Research article

A rapid assessment for predicting drug-induced hepatotoxicity using zebrafish

Yun Zhang [a], Liwen Han [a], Qiuxia He [a], Weiyun Chen [a], Chen Sun [a], Xue Wang [a], Xiqiang Chen [a], Rongchun Wang [a], Chung-Der Hsiao [b], Kechun Liu [a,*]

[a] Biology Institute of Shandong Academy of Sciences, Key Laboratory for Drug Screening Technology of Shandong Academy of Sciences, Shandong Provincial Engineering Laboratory for Biological Testing Technology, Key Laboratory for Biosensor of Shandong Province, 19 Keyuan Road, Lixia District, Jinan 250014, Shandong Province, PR China
[b] Department of Bioscience Technology, Chung Yuan Christian University, Chung-Li, 32023, Taiwan

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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

* Corresponding author at: Biology Institute of Shandong Academy of Sciences, 19 Keyuan Road, Lixia District, Jinan 250014, Shandong Province, PR China.
E-mail address: hliukch@sdas.org (K. Liu).

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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

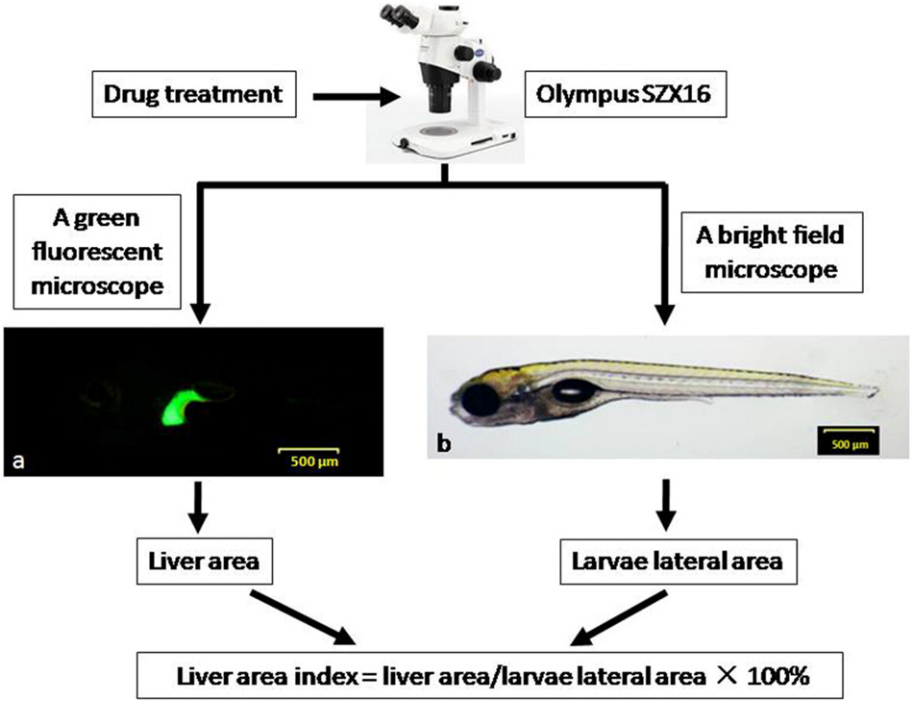

Fig. 1. The flow chart of the liver area index determination method. (a) The measurement of liver area. (b) The measurement of larvae lateral area.

are represented as the mean ± SD and are normally distributed. The significance of the differences between the groups was analyzed using one-way analysis of variance (ANOVA) and Dunnett's *t*-test. Differences were considered significant at $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Zebrafish liver development shows individual differences

The development of a functional liver in zebrafish is very rapid. By two days post-fertilization (dpf), liver tissue is easily recognized and the period of primary liver morphogenesis begins; the liver is perfused with blood and becomes fully functional by 3 dpf (Driessen et al., 2015) (Fig. 2A). However, individual differences occurred in the liver development of zebrafish (Fig. 2B).

3.2. The CV for liver area index

We examined the liver area and liver area index of 30 zebrafish in different development periods (3, 4, 5, 6, and 7 dpf). The change in liver area index as a percentage of the fish over time was demonstrated in Fig. 3. The range of CV for liver area was 17.28%–30.86%, and the range of CV for liver area index was 5.23%–11.13%. The CV for liver area index was significantly lower than the CV for liver area ($P < 0.01$), indicating the deviation of the liver area index was significantly smaller than the deviation of liver area (Table 1).

3.3. Quantitative image analysis of hepatotoxicity

After treatment with hepatotoxic drugs, the liver fluorescence and total fluorescence intensity of the zebrafish were significantly reduced (Fig. 4). In the 5 μM carbaryl, 4 mM isoniazide and 1 mM pyrazinamide groups, slightly swimming bladder diminution could be seen in some larvae (white solid arrowheads, Fig. 4). In the 10, 15, 20 μM carbaryl,8, 16 mM isoniazide and 2.5, 5 mM pyrazinamide groups, malformation became more severe, including absence of swimming bladder (red solid arrowheads, Fig. 4), severely yolk retention (red dotted arrowheads, Fig. 4) and liver degeneration (red circle indicates the liver, Fig. 4). Pericardial edema was found in zebrafish treated with 20 μM carbaryl, 16 mM isoniazide and 5 mM pyrazinamide (black solid arrowheads, Fig. 4).

We quantitatively assessed hepatotoxic drug-induced liver area index change in zebrafish (Fig. 5). Zebrafish larvae that were treated with 10, 15 and 20 μM carbaryl showed the liver area index level to be significantly decreased compared with the control group ($P < 0.05$). However, no apparent changes were observed in larvae treated with 5 μM carbaryl ($P > 0.05$). The liver area index levels in control and 5, 10, 15, 20 μM carbaryl groups were 4.28%, 3.93%, 2.02%, 1.31% and 0.98%, respectively. Zebrafish larvae treated with the middle and high doses of isoniazide (8 and 16 mM) and pyrazinamide (2.5 and 5 mM) showed the liver area index level to be significantly decreased compared with the control ($P < 0.01$). No apparent changes were observed in larvae treated with the low doses of isoniazide (4 mM) and pyrazinamide (1 mM) ($P > 0.05$). The liver area index levels in control and 4, 8, 16 mM isoniazide groups were 4.09%, 3.80%, 2.67 and 1.93%, respectively. And the liver area index levels in control and 1, 2.5, 5 mM pyrazinamide groups were 3.87%, 3.71%, 1.99% and 1.55%, respectively.

3.4. Histological analysis

To confirm that the significantly changed liver area index level truly represents liver damage, we evaluated the liver histopathology of the larvae treated with hepatotoxic drugs. The untreated and vehicle (0.5% DMSO)-treated zebrafish livers had the structural integrity of the hepatic cell. Loose cell-to-cell contact and large vacuoles can be seen in larvae treated with the middle and high doses of carbaryl (10, 15, and 20 μM), isoniazide (8 and 16 mM), and pyrazinamide (2.5 and 5 mM). The liver tissue damage degree increased with the increase of drug delivery dosage. Furthermore, the significantly decrease in the number of hepatocyte nucleus was observed in the larvae treated with 16 mM isoniazide. No apparent changes were observed in larval

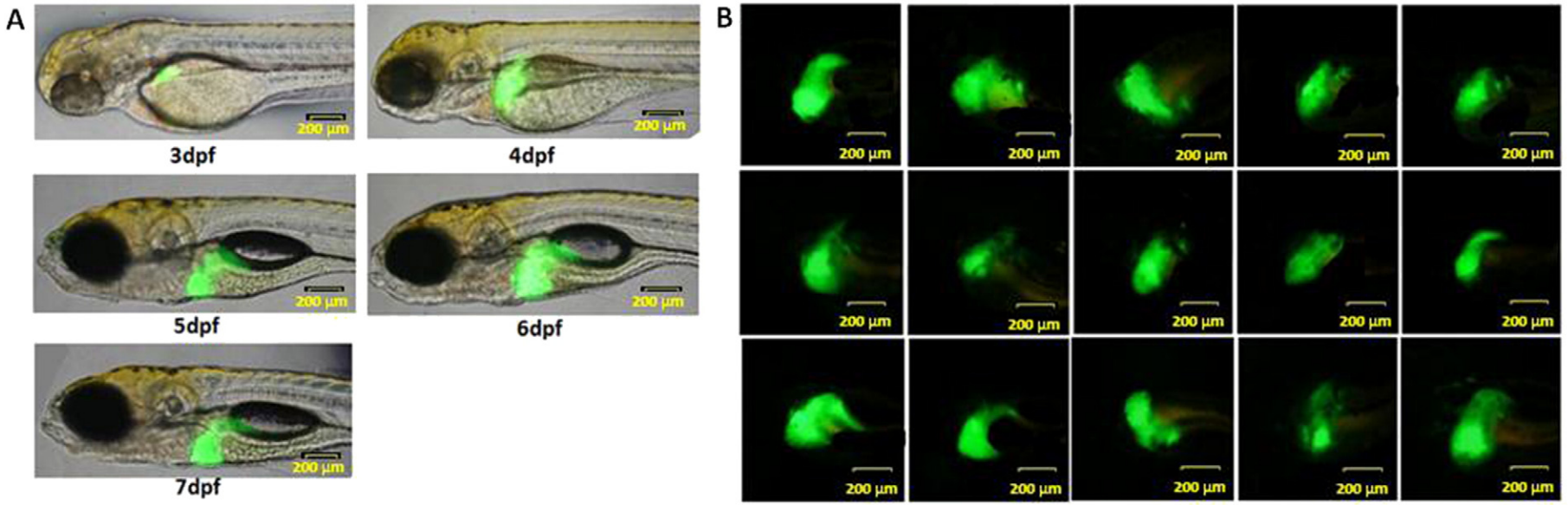

Fig. 2. In vivo imaging of larval L-FABP: EGFP zebrafish liver. (A) The liver morphology of zebrafish larvae in different development periods (3, 4, 5, 6, and 7 dpf). (B) Individual differences in liver development in zebrafish larvae at 5 dpf.

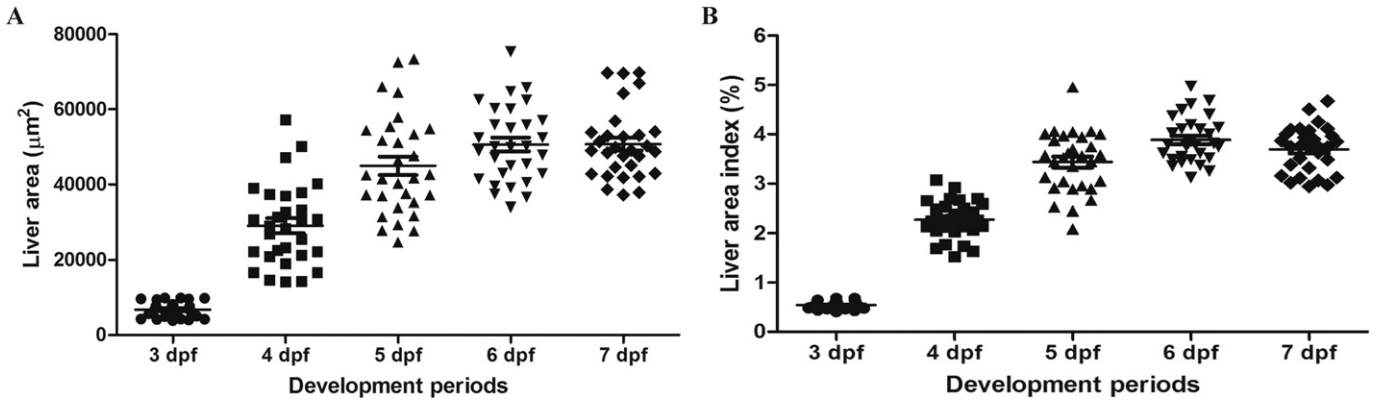

Fig. 3. The dispersions of liver area (A) and liver area index (B) in different development periods (3, 4, 5, 6, and 7 dpf) ($n = 30$).

hepatocytes treated with low doses of the drugs (5 μM carbaryl, 4 mM isoniazide, and 1 mM pyrazinamide) (Fig. 6).

3.5. ALT and AST activities in liver homogenates

To confirm that the significantly changed liver area index level truly represents liver damage, we determined the hepatic transaminase activities of the larvae treated with hepatotoxic drugs. ALT levels were significantly higher in the zebrafish larvae treated with 10 μM ($P < 0.05$), 15 μM ($P < 0.05$), and 20 μM ($P < 0.01$) of carbaryl, 8 mM ($P < 0.05$) and 16 mM ($P < 0.01$) of isoniazide, 2.5 mM ($P < 0.01$) and 5 mM ($P < 0.01$) of pyrazinamide, compared with the control. AST levels were also significantly higher in the zebrafish larvae treated with 10 μM ($P < 0.05$), 15 μM ($P < 0.05$), and 20 μM ($P < 0.01$) of carbaryl, 8 mM ($P < 0.05$) and 16 mM ($P < 0.01$) of isoniazide, 2.5 mM ($P < 0.05$) and 5 mM ($P < 0.05$) of pyrazinamide, compared with the control. No apparent changes were observed in larval hepatocytes treated with low doses of the drugs (5 μM carbaryl, 4 mM isoniazide, and 1 mM pyrazinamide) (Fig. 7).

3.6. Correlation between liver area index level and hepatic transaminase activities

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. The results showed a significant negative correlation between the degree of liver injury and the liver area index level. The liver area index level had the strong inverse correlation ($|r| > 0.7$, $P < 0.01$) with ALT and AST levels (Fig.8).

4. Discussion

Zebrafish have been used for rapid and high-throughput screening of compounds or drugs to evaluate hepatotoxicity and mechanisms of action (Driessen et al., 2013; Nam et al., 2016). Good correlation with mammalian hepatotoxicity has been observed (Driessen et al., 2014; Driessen et al., 2015; Verstraelen et al., 2016). Zebrafish complete primary liver morphogenesis by 2 dpf, and the liver is fully formed and functioning by 3 dpf (Isogai, Horiguchi, & Weinstein, 2001; Alderton et al., 2010). The development of a physiologically functional liver is very rapid, in comparison with other vertebrate models (McGrath, 2012). At approximately embryonic day 10 (ED 10) in the rat, the liver develops from the ventral foregut endoderm and acquires their polygonal structural characteristic just prior to birth (ED 20) (Hayashi et al., 2008; Mansuroglu, Dudas, Elmaouhoub, Joza, & Ramadori, 2009). Therefore, zebrafish is a advantageous model for assessing the liver injury of drugs. Hepatotoxicity of drugs is presently quantitatively assessed in larval zebrafish using changes in liver size. However, individual differences occurred in the liver development of zebrafish. Due to the different individual development speeds, the liver areas of individuals in the same developmental stages are quite different in the untreated zebrafish.

Our results showed that the CV for the liver area index was significantly lower than the CV for the liver area. The CV reflected the variation degree of the detection index. The smaller CV indicates a smaller deviation degree and greater accuracy of the determination results (Wong et al., 2016). These results indicated that the liver area index is more stable with smaller discrete degrees, higher precision, and better reproducibility. Using the liver index area to determine a significant alteration in liver size could reduce the number of animals needed compared to just liver area.

To confirm that the significantly changed liver area index level truly represents liver damage, we determined hepatic transaminase activities and evaluated the liver histopathology of the zebrafish larvae treated with the known mammalian hepatotoxic drugs carbaryl, isoniazide, and pyrazinamide. Carbaryl is a broad spectrum pesticide that is widely used as a contact and systemic insecticide on agricultural products (Gupta, Pillai, & Parmar, 2015) and is reported to cause histopathologic damages in liver tissues of *B. variabilis* (Cakici, 2015). Isoniazide and pyrazinamide are first-line drugs in tuberculosis combination chemotherapy. The principal side effects of isoniazide and pyrazinamide treatments are hepatic reactions (Babalik et al., 2012; Ramappa & Aithal, 2013). ALT and AST levels are standard tests in clinical practice for hepatotoxicity (Bouguezza, Khettal, Tir, & Boudrioua, 2015), and also are widely used to indicate zebrafish liver damage (Zhang, Li, & Gong, 2014a; Zhang et al., 2016). Zebrafish larvae treated with high doses of carbaryl, isoniazide, and pyrazinamide showed progressively decreased liver area index levels, suggestive of liver injury and corresponding to the higher hepatic transaminase activities and liver histological alterations. The liver area index levels of larvae treated with lowest observable adverse effect concentration (LOAEC) doses of drugs were not

Table 1
The CV for liver area and liver area index in different development period (3, 4, 5, 6, and 7 dpf).

	CV (%) for liver area	CV (%) for liver area index
3 dpf	30.86 ± 3.37	11.13 ± 2.41**
4 dpf	26.83 ± 2.63	6.4 ± 1.03**
5 dpf	21.92 ± 1.33	5.61 ± 0.85**
6 dpf	17.44 ± 2.76	5.23 ± 0.27**
7 dpf	17.28 ± 1.39	6.53 ± 0.86**

** $P < 0.01$ compared to CV for liver area at the same development period.

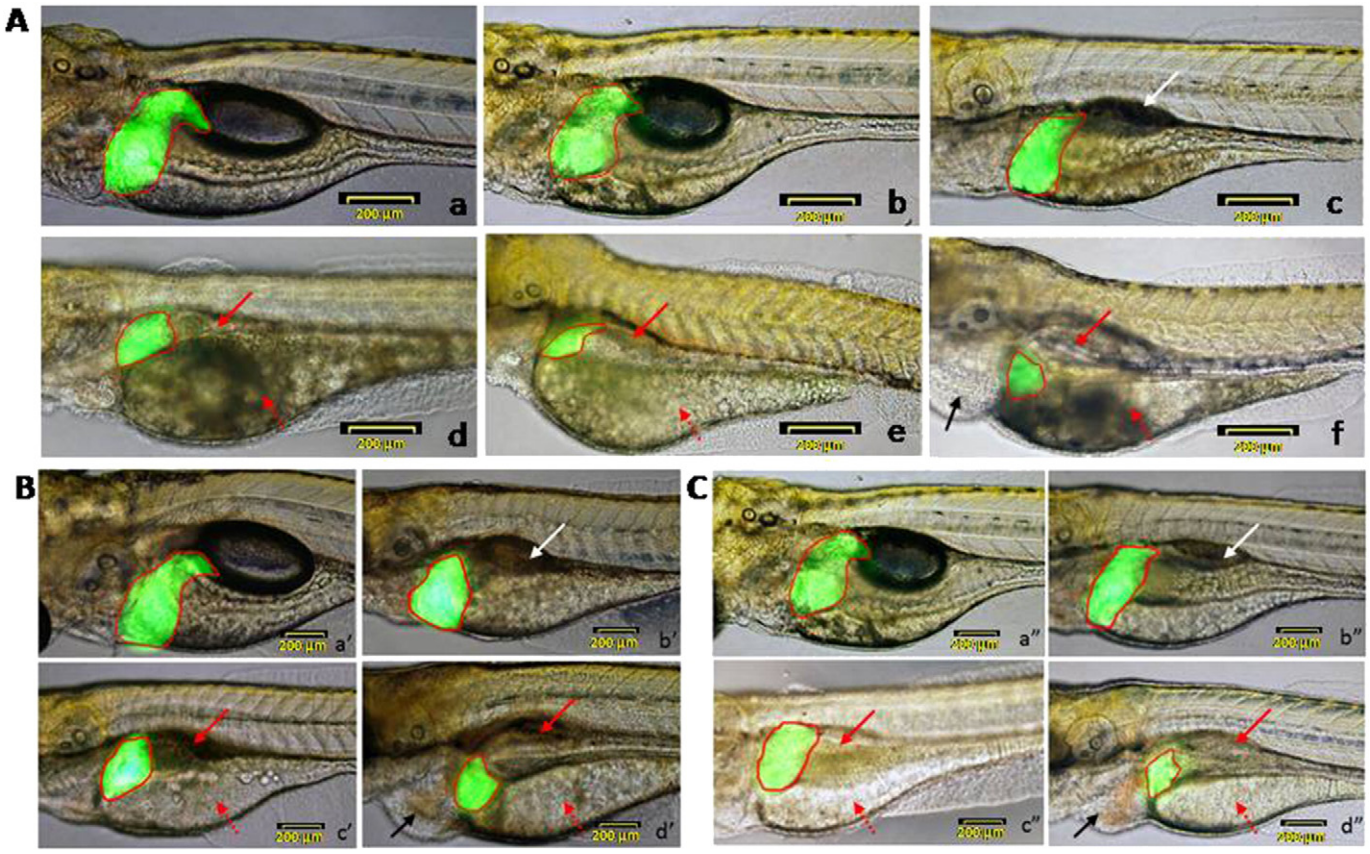

Fig. 4. In vivo visualization of liver morphology in larval L-FABP: EGFP zebrafish treated with (A) carbaryl, (B) isoniazide, or (C) pyrazinamide at 72 h post-exposure (hpe). a: control; b: 0.5% DMSO; c: 5 μM carbaryl; d: 10 μM carbaryl; e: 15 μM carbaryl; f: 20 μM carbaryl; a′: control; b′: 4 mM isoniazide; c′: 8 mM isoniazide; d′: 16 mM isoniazide; a″: control; b″: 1 mM pyrazinamide; c″: 2.5 mM pyrazinamide; d″: 5 mM pyrazinamide; Liver was indicated by red circle. The swim bladder diminution was indicated by white solid arrowheads. The swim bladder deficiency was indicated by red solid arrowheads. The yolk retention was indicated by red dotted arrowheads. Pericardial edema was indicated by black solid arrowheads.

significantly changed compared with the control, suggestive of no or small effect on the liver and corresponding to the normal hepatic transaminase activities and liver histopathology. The results demonstrated that our prediction method for evaluating hepatotoxic drugs using the liver area index of zebrafish was successful. The screening assay using liver area index can highly and rapidly predictive the compound hepatotoxicity, and could be combined with other markers for overt toxicity (acridine orange for example) to determine the liver damage mechanism of drugs.

The liver of zebrafish larvae is fairly globular in 3D structure. Different liver shapes were observed in different orientations of each fish. Hence, we kept the fish in the same orientation when the liver fluorescence and the whole larval lateral view were observed and photographed. All the larvae were fixed on the slide in a lateral view (two eyes were overlap) using 3% methyl cellulose after anesthetizing. In our study, the data of liver area index is based on 2D imaging. If the 3D image of liver is performed, the precision of detection is expected to be higher. However, 3D images and analyses are time- and cost-

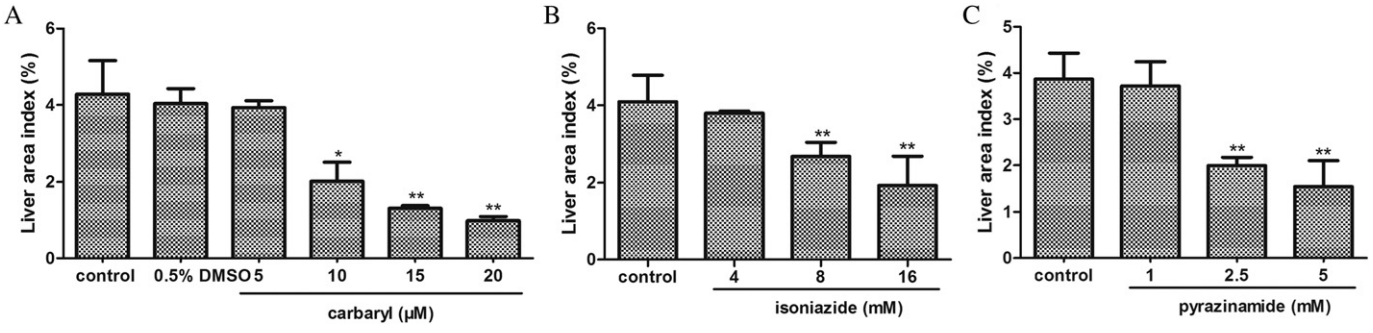

Fig. 5. Changes in the liver area index levels in zebrafish larvae treated with (A) carbaryl, (B) isoniazide, or (C) pyrazinamide at 72 hpe. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

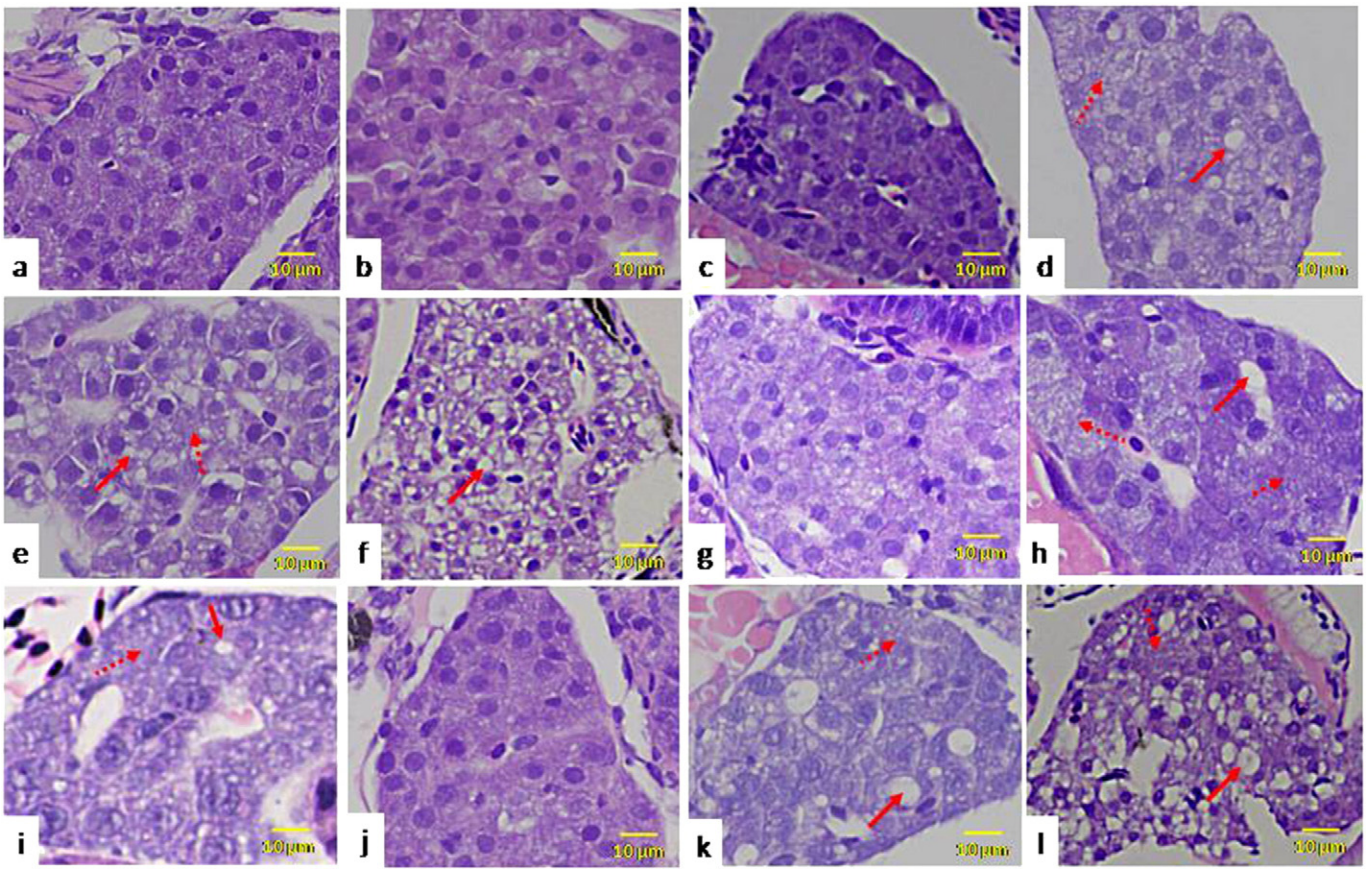

Fig. 6. Representative liver histological pictures of zebrafish larvae treated with (a) control, (b) 0.5% DMSO, (c) 5 μM carbaryl, (d) 10 μM carbaryl, (e) 15 μM carbaryl, (f) 20 μM carbaryl, (g) 4 mM isoniazide, (h) 8 mM isoniazide, (i) 16 mM isoniazide, (j) 1 mM pyrazinamide, (k) 2.5 mM pyrazinamide, or (l) 5 mM pyrazinamide at 72 hpe. Large vacuoles are indicated by red solid arrowheads. Loose cell-to-cell contact is indicated by red dotted arrowheads.

consuming, and are not suitable for high throughput screening. The screening assay using liver area index, a 2D imaging systems, is currently appropriate for a high throughput system.

In conclusion, the transgenic zebrafish Tg (L-FABP: EGFP), which expresses EGFP in the liver, was utilized to explore potential hepatotoxic effects of chemicals. This validated method provides a sensitive,

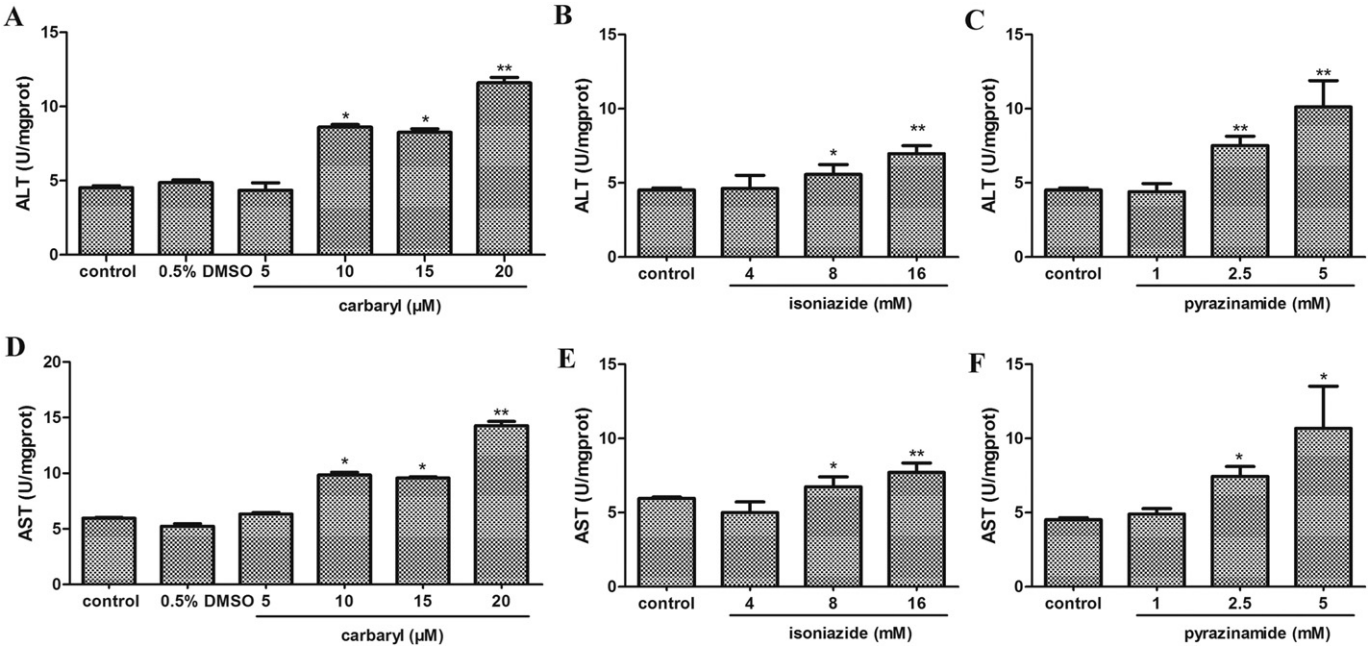

Fig. 7. Changes in hepatic transaminase activities in zebrafish larvae treated with hepatotoxic drugs at 72 hpe. (A, B and C) ALT levels in zebrafish larvae treated with carbaryl, isoniazide, and pyrazinamide. (D, E and F) AST levels in zebrafish larvae treated with carbaryl, isoniazide, and pyrazinamide. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

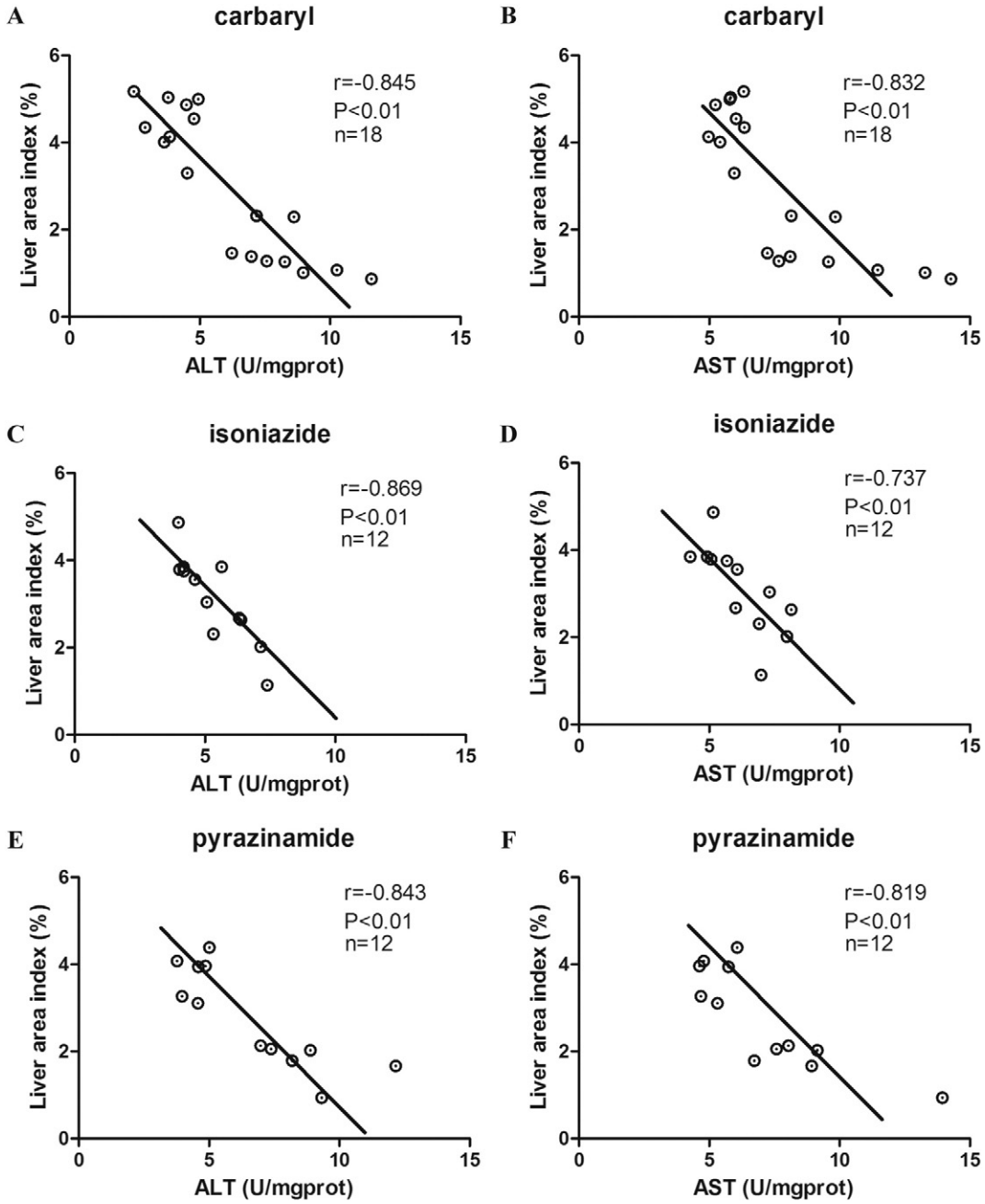

Fig. 8. The correlation between the liver area index level and hepatic transaminase activities in zebrafish treated with hepatotoxic drugs. (A) The liver area index level versus ALT activity in carbaryl-treated zebrafish. (B) The liver area index level versus AST activity in carbaryl-treated zebrafish. (C) The liver area index level versus ALT activity in isoniazide-treated zebrafish. (D) The liver area index level versus AST activity in isoniazide-treated zebrafish. (E) The liver area index level versus ALT activity in pyrazinamide-treated zebrafish. (F) The liver area index level versus AST activity in pyrazinamide-treated zebrafish. r represents the Pearson correlation coefficient.

efficient, and robust procedure to screen hepatotoxic chemicals at an earlier time point using liver area index, a new endpoint of hepatotoxicity.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

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References

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., ... Gardner, I. (2010). Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. *Xenobiotica*, *40*(8), 547–557.

Babalik, A., Arda, H., Bakirci, N., Agca, S., Oruc, K., Kiziltas, S., ... Calisir, H. C. (2012). Management of and risk factors related to hepatotoxicity during tuberculosis treatment. *Tüberküloz ve Toraks*, *60*(2), 136–144.

Bissell, D. M., Gores, G. J., Laskin, D. L., & Hoofnagle, J. H. (2001). Drug-induced liver injury: Mechanisms and test systems. *Hepatology*, *33*(4), 1009–1013.

Bouguezza, Y., Khettal, B., Tir, L., & Boudrioua, S. (2015). Damascenine induced hepatotoxicity and nephrotoxicity in mice and in vitro assessed human erythrocyte toxicity. *Interdisciplinary Toxicology*, *8*(3), 118–124.

Cakici, O. (2015). Histopathologic changes in liver and kidney tissues induced by carbaryl in *Bufotes variabilis* (Anura: Bufonidae). *Experimental and Toxicologic Pathology*, *67*(3), 237–243.

Driessen, M., Kienhuis, A. S., Pennings, J. L., Pronk, T. E., van de Brandhof, E. J., Roodbergen, M., ... van der Ven, L. T. (2013). Exploring the zebrafish embryo as an alternative model for the evaluation of liver toxicity by histopathology and expression profiling. *Archives of Toxicology*, *87*(5), 807–823.

Driessen, M., Kienhuis, A. S., Vitins, A. P., Pennings, J. L., Pronk, T. E., van den Brandhof, E. J., ... van der Ven, L. T. (2014). Gene expression markers in the zebrafish embryo reflect a hepatotoxic response in animal models and humans. *Toxicology Letters*, *230*(1), 48–56.

Driessen, M., Vitins, A. P., Pennings, J. L., Kienhuis, A. S., Water, B., & van der Ven, L. T. (2015). A transcriptomics-based hepatotoxicity comparison between the zebrafish embryo and established human and rodent in vitro and in vivo models using cyclosporine A, amiodarone and acetaminophen. *Toxicology Letters*, *232*(2), 403–412.

Eun, J. W., Bae, H. J., Shen, Q., Park, S. J., Kim, H. S., Shin, W. C., ... Nam, S. W. (2015). Characteristic molecular and proteomic signatures of drug-induced liver injury in a rat model. *Journal of Applied Toxicology*, *35*, 152–164.

Field, H. A., Ober, E. A., Roeser, T., & Stainier, D. Y. (2003). Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Developmental Biology*, *253*, 279–290.

Fontana, R. J. (2014). Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology*, *146*(4), 914–928.

Frenzel, C., & Teschke, R. (2016). Herbal hepatotoxicity: Clinical characteristics and listing compilation. *International Journal of Molecular Sciences*, *17*(5). http://dx.doi.org/10.3390/ijms17050588.

Gamse, J. T., & Gorelick, D. A. (2016). Mixtures, metabolites, and mechanisms: Understanding toxicology using zebrafish. *Zebrafish*, *13*(5), 377–378.

Gomez-Lechon, M. J., Tolosa, L., & Donato, M. T. (2015). Metabolic activation and drug-induced liver injury: in vitro approaches for the safety risk assessment of new drugs. *Journal of Applied Toxicology*, *36*(6), 752–768.

Gupta, N., Pillai, A. K., & Parmar, P. (2015). Spectrophotometric determination of trace carbaryl in water and grain samples by inhibition of the rhodamine-B oxidation. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*, *139*, 471–476.

Hayashi, Y., Toda, K., Saibara, T., Okamoto, S., Osanai, M., Enzan, H., & Lee, G. H. (2008). Expression of fascin-1, an actin-bundling protein, in migrating hepatoblasts during rat liver development. *Cell and Tissue Research*, *334*(2), 219–226.

He, J. H., Guo, S. Y., Zhu, F., Zhu, J. J., Chen, Y. X., Huang, C. J., ... Li, C. Q. (2012). A zebrafish phenotypic assay for assessing drug-induced hepatotoxicity. *Journal of Pharmacological and Toxicological Methods*, *67*(1), 25–32.

Her, G. M., Chiang, C. C., Chen, W. Y., & Wu, J. L. (2003). In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (*Danio rerio*). *FEBS Letters*, *538*(1–3), 125–133.

Hill, A. J., Howard, C. V., & Cossins, A. R. (2002). Efficient embedding technique for preparing small specimens for stereological volume estimation: Zebrafish larvae. *Journal of Microscopy*, *206*(3), 179–181.

Hill, A., Mesens, N., Steemans, M., Xu, J. J., & Aleo, M. D. (2012). Comparisons between in vitro whole cell imaging and in vivo zebrafish-based approaches for identifying potential human hepatotoxicants earlier in pharmaceutical development. *Drug Metabolism Reviews*, *44*(1), 127–140.

Hinton, D. E., & Couch, J. A. (1998). Architectural pattern, tissue and cellular morphology in livers of fishes: relationship to experimentally-induced neoplastic responses. *Experientia. Supplementum*, *86*, 141–164.

Isogai, S., Horiguchi, M., & Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: An atlas of embryonic and early larval development. *Developmental Biology*, *230*(2), 278–301.

Johnson, S. L., & Zon, L. I. (1999). Genetic backgrounds and some standard stocks and strains used in zebrafish developmental biology and genetics. *Methods in Cell Biology*, *60*, 357–359.

Lee, H. J., Lowdon, R. F., Maricque, B., Zhang, B., Stevens, M., Li, D., ... Wang, T. (2015). Developmental enhancers revealed by extensive DNA methylome maps of zebrafish early embryos. *Nature Communications*, *6*, 6315.

Mansuroglu, T., Dudas, J., Elmaouhoub, A., Joza, T. Z., & Ramadori, G. (2009). Hepatoblast and mesenchymal cell-specific gene-expression in fetal rat liver and in cultured fetal rat liver cells. *Histochemistry and Cell Biology*, *132*(1), 11–19.

McGrath, P. (Ed.). (2012). *Zebrafish: Methods for assessing drug safety and toxicity*. John Wiley & Sons, Inc.

McGrath, P., & Li, C. Q. (2008). Zebrafish: A predictive model for assessing drug-induced toxicity. *Drug Discovery Today*, *13*(9–10), 394–401.

Mesens, N., Crawford, A. D., Menke, A., Hung, P. D., Van Goethem, F., Nuyts, R., ... Esguerra, C. V. (2015). Are zebrafish larvae suitable for assessing the hepatotoxicity potential of drug candidates? *Journal of Applied Toxicology*, *35*(9), 1017–1029.

Nam, H. S., Hwang, K. S., Jeong, Y. M., Ryu, J. I., Choi, T. Y., Bae, M. A., ... Kim, C. H. (2016). Expression of miRNA-122 Induced by Liver Toxicants in Zebrafish. *BioMed Research International*, *2016*, 1473578.

Norris, W., Paredes, A. H., & Lewis, J. H. (2008). Drug-induced liver injury in 2007. *Current Opinion in Gastroenterology*, *24*(3), 287–297.

North, T. E., Babu, I. R., Vedder, L. M., Lord, A. M., Wishnok, J. S., Tannenbaum, S. R., ... Goessling, W. (2010). PGE2-regulated wnt signaling and *N*-acetylcysteine are synergistically hepatoprotective in zebrafish acetaminophen injury. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(40), 17315–17320.

Ramappa, V., & Aithal, G. P. (2013). Hepatotoxicity related to anti-tuberculosis drugs: Mechanisms and management. *Journal of Clinical and Experimental Hepatology*, *3*(1), 37–49.

Sabaliauskas, N. A., Foutz, C. A., Mest, J. R., Budgeon, L. R., Sidor, A. T., Gershenson, J. A., ... Cheng, K. C. (2006). High-throughput zebrafish histology. *Methods*, *39*(3), 246–254.

Sarges, P., Steinberg, J. M., & Lewis, J. H. (2016). Drug-induced liver injury: Highlights from a review of the 2015 literature. *Drug Safety*, *39*(9), 801–821.

Sison-Young, R. L., Lauschke, V. M., Johann, E., Alexandre, E., Antherieu, S., Aerts, H., ... Park, B. K. (2016). A multicenter assessment of single-cell models aligned to standard measures of cell health for prediction of acute hepatotoxicity. *Archives of Toxicology* [Epub ahead of print], http://dx.doi.org/10.1007/s00204-016-1745-4.

Teo, D. C., Ng, P. S., Tan, S. H., Lim, A. T., Toh, D. S., Chan, S. Y., & Cheong, H. H. (2016). Drug-induced liver injury associated with complementary and alternative medicine: A review of adverse event reports in an Asian community from 2009 to 2014. *BMC Complementary and Alternative Medicine*, *16*(1), 192.

Verstraelen, S., Peers, B., Maho, W., Hollanders, K., Remy, S., Berckmans, P., ... Witters, H. (2016). Phenotypic and biomarker evaluation of zebrafish larvae as an alternative model to predict mammalian hepatotoxicity. *Journal of Applied Toxicology*, *36*(9), 1194–1206.

Vliegenthart, A. D., Tucker, C. S., Del Pozo, J., & Dear, J. W. (2014). Zebrafish as model organisms for studying drug-induced liver injury. *British Journal of Clinical Pharmacology*, *78*(6), 1217–1227.

Wong, J., Davies, N., Jeraj, H., Vilar, E., Viljoen, A., & Farrington, K. (2016). A comparative study of blood endotoxin detection in haemodialysis patients. *Journal of Inflammation (Lond)*, *13*, 24.

Yang, F., Chen, Z., Pan, J., Li, X., Feng, J., & Yang, H. (2011). An integrated microfluidic array system for evaluating toxicity and teratogenicity of drugs on embryonic zebrafish developmental dynamics. *Biomicrofluidics*, *5*(2), 24115.

Zhang de, L., Liu, S. Y., Zhang, J., Zhang, J. K., Hu, C. X., & Liu, Y. D. (2016). Respiratory toxicity of cyanobacterial aphantoxins from Aphanizomenon flos-aquae DC-1 in the zebrafish gill. *Aquatic Toxicology*, *176*, 106–115.

Zhang, X., Li, C., & Gong, Z. (2014a). Development of a convenient in vivo hepatotoxin assay using a transgenic zebrafish line with liver-specific DsRed expression. *PloS One*, *9*(3), e91874.

Zhang, D. L., Zhang, J., Hu, C. X., Wang, G. H., Li, D. H., & Liu, Y. D. (2014b). Morphological alterations and acetylcholinesterase and monoamine oxidase inhibition in liver of zebrafish exposed to Aphanizomenon flos-aquae DC-1 aphantoxins. *Aquatic Toxicology*, *157*, 215–224.

Zhang, Y., Liu, K., Hassan, H. M., Guo, H., Ding, P., Han, L., ... Jiang, Z. (2016). L-FABP-deficiency provoked oxidative stress, inflammation and apoptosis-mediated hepatotoxicity induced by pyrazinamide on zebrafish larvae. *Antimicrobial Agents and Chemotherapy*, *60*(12), 7347–7356.