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Oxidative stress-mediated developmental toxicity induced by isoniazide in zebrafish embryos and larvae

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ABSTRACT: Isoniazide (INH) is an important first-line drug that is used to treat tuberculosis. However, the effect of INH on fetal growth has not yet been elucidated, and the mechanism of INH-induced developmental toxicity is still unknown. In the present study, we employed zebrafish embryos and larvae to investigate the developmental toxicity of INH. The survival rates of the embryos and larvae as well as the hatching rates of embryos were significantly reduced. Morphological abnormalities, including spinal curvature, yolk retention, swimming bladder absence, tail bending and shorter body lengths were induced by INH. Histopathological analysis showed loose cell-to-cell contacts and large vacuoles in the larval hepatocytes. Thin intestinal walls, frayed gut villi and widespread cell lysis were observed in the intestines of the larvae in the higher concentration (8, 16 mM) exposure groups. In addition, exposure to high doses (≥ 6 mM) of INH significantly reduced the locomotor capacity of the zebrafish larvae. INH significantly increased the levels of reactive oxygen species and malondialdehyde and decreased the superoxide dismutase activity in zebrafish larvae, which suggested that oxidative stress was induced and that the antioxidant capacity was inhibited. Superoxide dismutase 1 and liver fatty acid-binding protein mRNA levels were significantly downregulated, while the GSTP2 and cytochrome P450 3A mRNA levels were significantly upregulated in the INH-exposed zebrafish larvae. The overall results indicated that INH caused a dose- and time-dependent increase in developmental toxicity and that oxidative stress played an important role in the developmental toxicity induced by INH in zebrafish larvae. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: Isoniazide; developmental toxicity; zebrafish embryos; zebrafish larvae; swimming behavior; oxidative stress

Introduction

Tuberculosis (TB) is a disease that has a serious impact on public health and has a high incidence in China (Negin *et al.*, 2016). The disease causes approximately 1.5 million deaths per year worldwide (Taylor *et al.*, 2015). Isoniazide (INH) has been a first-line drug in the clinical treatment of TB for many decades (Khan *et al.*, 2016). For INH, several hypotheses have been proposed to account for its efficacy against *Mycobacterium tuberculosis* growth. The most accepted mechanism of action proposes INH inhibits mycolic acid biosynthesis as a pro-drug (Campen *et al.*, 2015). INH has many advantages as a treatment including a wide antibacterial effect, good biological membrane penetrability, superior curative effect and low price (Bernardes-Genisson *et al.*, 2013). INH can provide a maximum of approximately 90% protective efficacy in TB. However, INH leads to some adverse effects that include hepatitis, peripheral neurotoxicity, lupus-like syndrome, hypersensitivity reactions and rare but serious complications in the central nervous system (Hasiloglu *et al.*, 2012).

INH can cross the placental barrier, which can cause the fetal blood drug concentrations to be higher than those in the maternal blood can. A previous report showed that INH causes intrauterine fetal necrosis in pregnant women (Zolcinski, 1962). The occurrence of adverse reactions should be closely monitored in pregnant women and newborns that use INH. Despite the important role of INH in TB treatment, the effects of INH on the growth of the fetus have not yet been elucidated, and the mechanism for the INH-induced developmental toxicity is still unknown. Some scholars speculate that the pharmacological mechanism of INH action may involve its reactive metabolites (Ramappa & Aithal, 2013).

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Zebrafish are a widespread model in studies of ontogenetic developmental responses following exposure to a drug (McGrath & Li, 2008). The advantages of zebrafish as a toxicological model species include their small size, transparency during early life stages, ease of handling in an experimental setting, high fecundity, and rapid embryogenesis and organogenesis *in vitro*. These features make it feasible to detect the toxicity and the pathways involved in drug exposure. In addition, the genome of zebrafish is approximately 87% homologous to that of humans (Fraysse *et al.*, 2006). In mammalian systems, it has been confirmed that the toxicity of exogenous material on the fetal systems is antidotal by maternal systems, so it is very difficult to observe the very delicate changes during early embryonic development. The development of zebrafish embryos and larvae occurs outside of the mother, thus eliminating any maternal effects. Therefore, zebrafish are used as a universal model to investigate toxicity during the early stages of embryonic development (Haendel *et al.*, 2004).

In the present study, zebrafish embryos and larvae were employed to investigate the developmental toxicity induced by INH. The developmental abnormalities caused by INH included scoliosis, increased yolk sac areas, uninflated swim bladders, tail bending and shorter body lengths. The aims of this study were to investigate the mechanism for the developmental toxicity of INH and to provide valuable information for further assessments before clinical trials.

Materials and methods

Chemicals

INH was purchased from Sigma (St. Louis, MO, USA). The stock solutions were prepared in double-distilled water (ddH_2O), and serial dilutions were made in aquarium water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) before the experiments. All other chemicals and reagents utilized in this study were of analytical grade.

Zebrafish maintenance and embryo collection

Wild-type (AB strain) zebrafish were maintained at 28 ± 0.5°C in a 14 : 10 h light/dark cycle in a closed flow-through system with charcoal-filtered tap water. The fish were fed brine shrimp twice daily. The zebrafish embryos were obtained from spawning adults that were placed in groups of approximately 20 males and 10 females in tanks overnight. Spawning was induced in the morning when the light was turned on. At 4 h post-fertilization (hpf), the embryos were examined under a dissecting microscope (Motic, Xiamen, China), and the embryos that had developed normally to the blastula stage were selected for subsequent experiments (Sun *et al.*, 2013).

Zebrafish embryo toxicity

At 6 hpf, the normal embryos were selected under a stereomicroscope (Olympus SZX16, Tokyo, Japan). The zygotes were randomly distributed into six-well plates (30 per well). The 6 hpf embryos were exposed to various concentrations of INH (1, 2, 4, 6, 8, 16, 32 or 64 mM) dissolved in 5 ml of the fish water. Zebrafish treated with fish water were used as vehicle controls. The solutions were changed once every 24 h at which time any dead embryos were discarded. The exposure began at approximately 6 hpf and ended at approximately 72 hpf. All of the embryos were kept at 28 ± 0.5°C and subjected to an environment with a light and dark cycle. During the 72 h post-exposure (hpe), the embryos were examined under a stereomicroscope to observe morphological abnormalities. The hatching rates and mortality were recorded within each group at 24, 48 and 72 hpe.

Zebrafish larval toxicity

After 72 hpf, the normal larvae were selected under a stereomicroscope. The larvae were randomly distributed into six-well plates (15 per well) and exposed to a range of INH concentrations (1, 2, 4, 6, 8 and 16 mM) dissolved in 5 ml of the fish water. Zebrafish treated with fish water were used as vehicle controls. The dose range was selected according to values previously ascertained in range-finding studies, which identified the concentration ranged from no effect on the development to clear the toxic effect on development in a short exposure period (Ankley *et al.*, 2005; Kannan *et al.*, 2005). The study was carried out in triplicate. The solutions were changed once every 24 h, and the dead larvae were removed. The experimental temperature was maintained at 28 ± 0.5°C. The development of the zebrafish larvae was monitored at 24, 48 and 72 hpe. During the 72 h exposure, the toxicity as indicated by mortality, malformation rates and changes in the body lengths was recorded at 24 h intervals. Mortality was identified by coagulation of the larvae, missing heartbeat and a non-detached tail. Each group of zebrafish larvae was examined with a stereomicroscope to screen for morphological abnormalities. The length of each zebrafish larva along the body axis from the anterior-most part of the head to the tip of the tail was measured from these digital images using Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Behavioral analysis

Behavioral testing was performed at 24, 48 and 72 hpe. The larvae from each group were collected, cleaned in fish water and placed in 24-well plates. Each well contained 2 ml of fish water and one larva; there were six larvae in each group. After a 15 min acclimation period, the larvae were allowed to explore the well freely. A camera positioned above the plate was used to track movement for 10 min. Zeblab software (Viewpoint, Lyon, France) was used to analyze the digital tracks, and the average speed was analyzed every 30 s.

Histopathological evaluations

After the drug treatment, the larval zebrafish were fixed in 4% paraformaldehyde. For the histopathological examinations, all of the fixed larvae were processed by embedding in paraffin, sectioning and staining with hematoxylin and eosin.

Measurement of reactive oxygen species generation

After drug treatment, the generation of reactive oxygen species (ROS) in the larval zebrafish was analyzed using 2′,7′-dichlorodihydrofluorescein diacetate. The larvae were transferred to 24-well plates, treated with a solution of 20 μg ml^{-1} 2′,7′-dichlorodihydrofluorescein diacetate and incubated for 1 h in the dark at 28°C. After this incubation, the larvae were washed with fresh fish water and anesthetized with 0.16% Tricaine. Images of the individual larvae were collected using a fluorescence

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microscope (Olympus), and the fluorescence intensity was quantified using the Image J program.

Determinations of oxidative stress-related parameters

For each condition, 50 zebrafish larvae were collected after the INH treatments and homogenized on ice in cold saline. The homogenates were centrifuged at 2500 rpm (615 g) at 4°C for 10 min to obtain the supernatants. The malondialdehyde (MDA) levels and the superoxide dismutase (SOD) activities in these samples were determined using their respective commercial kits (Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturer's protocols.

Total RNA extraction and reverse transcription–polymerase chain reaction

The total RNA was extracted from 30 homogenized zebrafish larvae (exposed for 24, 48 and 72 hpe) using the NanoMag Animal and Fish RNA Isolation Kit (Shannuo Scientific Company, Tianjin, China). The total RNA contents were determined by measuring the absorbance at 260 nm, and the quality was verified by measuring the 260/280 nm ratio. The synthesis of cDNA was performed using 1 μg of total RNA from each sample mixed with 1 μl of random primers (Takara, Kyoto, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with SYBR-Green using the iTaq™ universal SYBR® Green supermix (Bio-Rad, Foster, CA, USA) and a Bio-Rad CFX96 Real-Time System. The thermal cycler conditions included holding for 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 10 s at 60°C. The target genes and the β-actin reference gene in all samples were evaluated using fluorescence quantitative PCR to obtain the respective C_T values; the relative gene expression differences in each sample were analyzed using the $2^{-\Delta\Delta CT}$ method. The reverse transcription–PCR reactions for each selected gene were performed on three replicate samples. The genes that were detected and their primer sequences are shown as Table 1.

Statistical analyses

The data are presented as the means ± standard error. The data analyses were performed using the SPSS software package. Significant differences between the mean values were determined using one-way analysis of variance and Dunnett's *t*-test. The differences were considered statistically significant at $^*P < 0.05$ or $^{**}P < 0.01$.

Table 1. Quantitative polymerase chain reaction primer sequences

Gene	Primer orientation	Nucleotide sequence
β-actin	Forward	5′-AGAGCTATGAGCTGCCTGACG-3′
	Reverse	5′-CCGCAAGATTCCATACCCA-3′
L-FABP	Forward	5′-ACGTGGCAGGTTTACGCTCAG-3′
	Reverse	5′-TTGGAGGTGATGGTGAAGTCG-3′
SOD1	Forward	5′-GGCCAACCGATAGTGTTAGA-3′
	Reverse	5′-CCAGCGTTGCCAGTTTTTAG-3′
GSTP2	Forward	5′-CACAGACCTCGCTTTTCACAC-3′
	Reverse	5′-GAGAGAAGCCTCACAGTCGT-3′
CYP3A	Forward	5′-GGTGGAGGAGATCGACAA AA-3′
	Reverse	5′-ACCGTTTTCTTAGCGGACCT-3′

Results

Developmental toxicity effect of isoniazide in zebrafish embryos

Mortality rate. The mortality rates for the embryos after the INH treatments are shown in Fig. 1(A). Significantly increased mortality was observed at 24 and 48 hpe for concentrations higher than 16 mM. Furthermore, significant increases in mortality became apparent by 72 hpe at concentrations higher than 6 mM. The death rate following exposure to 64 mM INH (56.67 ± 6.67%, $P < 0.01$) was significantly increased compared with the control group (10.00 ± 3.33%) by 72 hpe.

Hatching rate. Hatching is known to be a critical period in zebrafish embryogenesis. In the control group and in the groups exposed to various concentrations of INH, the embryos began to hatch at 48 hpe and finished by 96 hpe, and all the surviving embryos had hatched by the end of the experiment. The data demonstrated that 70% and 90% of the control zebrafish embryos had hatched by 48 and 72 hpe, respectively, while the embryo-hatching rate was significantly reduced in the groups treated with INH at concentrations higher than 4 mM (Fig. 1B). Only sporadic embryo hatching was observed in the group incubated with 64 mM of INH. These data demonstrated a remarkable dose- and time-dependent decrease in the hatching rate in the INH-treated groups compared with that of the control.

Malformations. Figure 1(C) shows the development of the zebrafish embryos that were exposed to INH from 6 to 72 hpf. At 24 hpe, no malformations were observed in the INH-treated groups (1, 2, 4, 6, 8, 16, 32 mM), although a slight developmental delay was observed in the 64 mM INH-treated embryos. At 48 hpe, there were significant differences in the malformation phenotypes in the zebrafish embryos that were exposed to the various doses of INH. Many of the zebrafish embryos in the control group had shed the membrane whereas the INH-treated (8, 16, 32 or 64 mM) groups showed a marked delay in hatching. At the subsequent times, malformations became apparent including the presence of edema and spinal curvature in the 16, 32 and 64 mM INH groups at 72 hpe. Not all of the 64 mM INH-treated embryos hatched, and severely abnormal morphogenesis including spinal curvature and edema was observed in the embryos.

Developmental toxicity effect of isoniazide in zebrafish larvae

Mortality rate. The percentage of mortality following exposure to INH was markedly increased in a dose- and time-dependent manner (Fig. 2A). There were no significant differences in the percentages of death in any of the exposed groups compared to the control group at 24 hpe. However, a significant increase in mortality at 48 hpe was found in the larvae that were exposed to 16 mM INH. The larvae exposed to 6, 8 or 16 mM INH showed a sharp increase in mortality at 72 hpe. The 16 mM concentration of INH induced approximately 85% mortality at 72 hpe.

Body length. The body lengths of the larvae were measured at 24, 48 and 72 hpe to assess growth (Fig. 2B). The body lengths of the larvae at 24 hpe were significantly reduced in the groups treated with 8 or 16 mM INH. At both 48 and 72 hpe, the body

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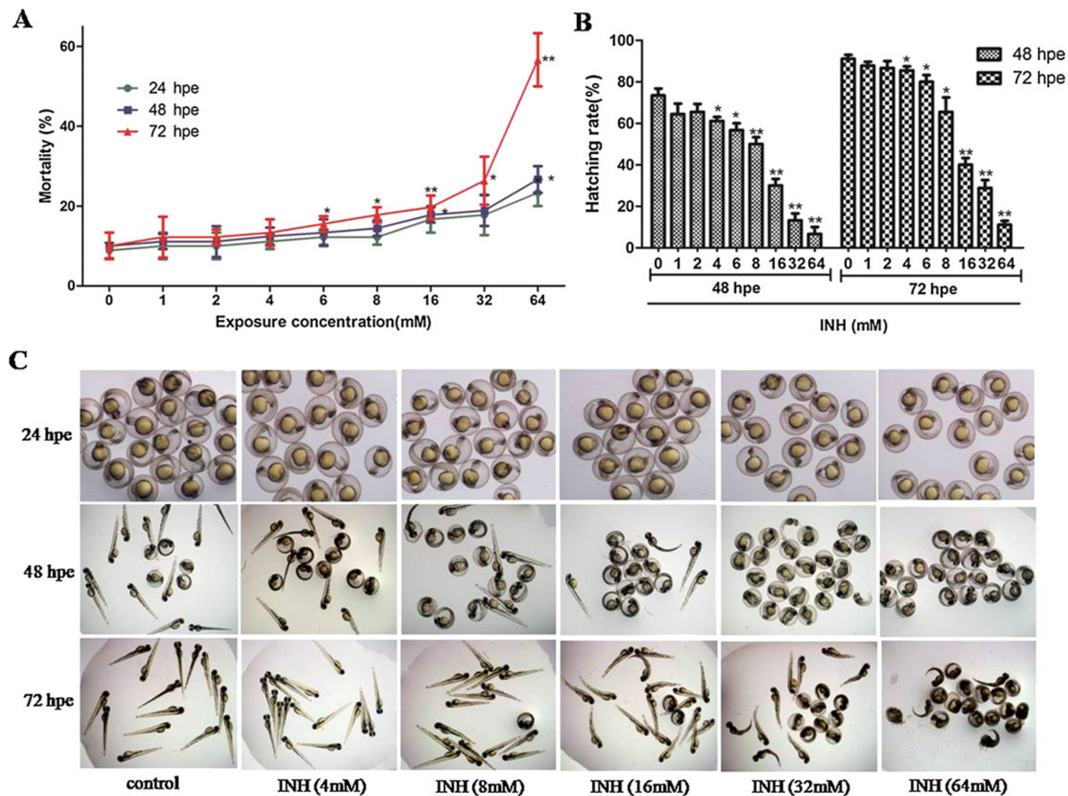

Figure 1. Developmental toxicity effects of INH in zebrafish embryos. (A) Mortality rates in INH-exposed zebrafish embryos at 24, 48 and 72 hpe. (B) Hatching rates at 48 and 72 hpf for zebrafish embryos exposed to INH. (C) Effects of INH on embryonic morphological changes at 24, 48 and 72 hpf. *$P < 0.05$, **$P < 0.01$ versus control. Hpe, hours post-exposure; hpf, hours post-fertilization; INH, isoniazide.

lengths of the groups treated with 2, 4, 6, 8 or 16 mM were significantly reduced compared with the control.

Malformation. The malformation rates were recorded in the larvae at 24, 48 and 72 hpe (Fig. 2C). In this study, the zebrafish larvae are assessed for morphological abnormalities with a numerical score system designed to distinguish the severity of the developmental toxicity effects observed in multiple organ systems of the zebrafish larvae after exposure to the specified INH doses (Fig. 3-E–G). The evaluation of the morphological endpoints of this assay involved assigning a score from 5 to 0.5 (with score values decreasing as the malformations increased) for the following structures: body shape, face, brain, somites, notochord, swim bladder, yolk sac, tail, fins and pharyngeal arches/jaws. The general morphological scoring criteria were described in the previous literature (Augustine-Rauch *et al.*, 2010; Panzica-Kelly *et al.*, 2010).

At 24 hpe, no significant malformations were observed for the samples exposed to 1, 2, 4 or 6 mM INH. The malformation rate was significantly increased in the 16 mM INH-treated group (56.67 ± 5.77%). Abnormalities including swim bladder deficiency and yolk retention were found in the 8 and 16 mM INH groups (Fig. 3A,B).

At 48 hpe, there were significant increases in the rates of malformation in the INH-treated groups compared with the control group, with the malformation rates of 31.67 ± 10.41%, 35.00 ± 5.00% and 92.22 ± 6.94% in the 6, 8 and 16 mM INH-treated groups, respectively (Fig. 2C). These INH exposures caused severe yolk retention, swim bladder absence and tail

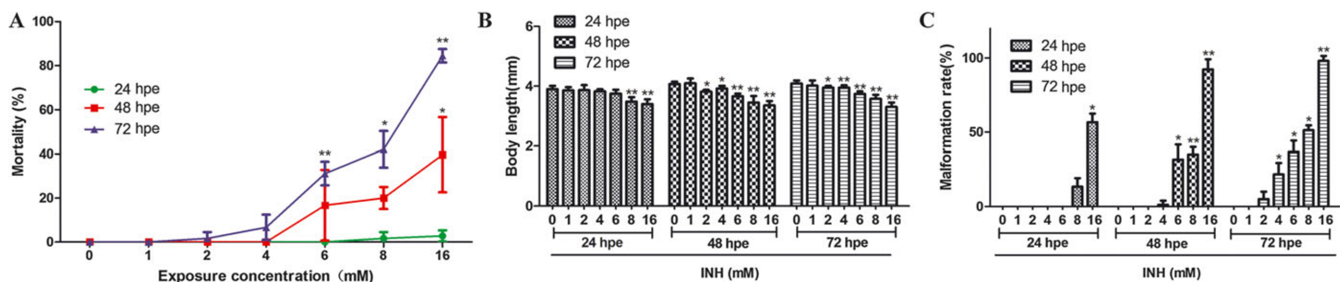

Figure 2. Developmental toxicity effect of INH in zebrafish larvae. (A) Mortality rates in INH-exposed zebrafish larvae at 24, 48 and 72 hpe. (B) Body lengths at 24, 48 and 72 hpe in zebrafish larvae exposed to INH. (C) Malformation rates at 24, 48 and 72 hpe in zebrafish larvae exposed to INH. *$P < 0.05$, **$P < 0.01$ versus control. Hpe, hours post-exposure; INH, isoniazide.

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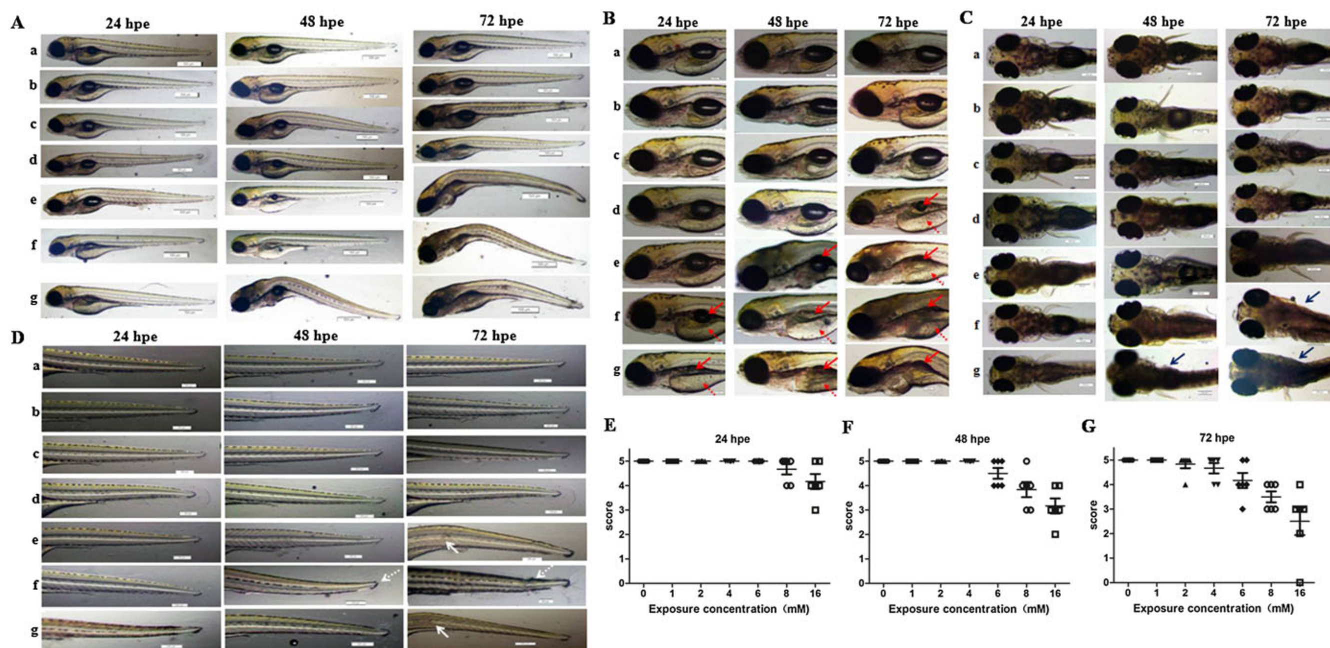

Figure 3. Body shape morphology of INH-exposed zebrafish larvae at 24, 48 and 72 hpe. Larvae were treated with: (a) 0, (b) 1, (c) 2, (d) 4, (e) 6, (f) 8 or (g) 16 mM INH. (A,B) Representative lateral views of zebrafish larvae. (C) Representative ventral views of zebrafish larvae. (D) Representative lateral views of the tails of zebrafish larvae. (E–G) General morphological scoring of INH-exposed zebrafish larvae at 24, 48 and 72 hpe. Swim bladder deficiency is indicated by solid red arrowheads. Yolk retention is indicated by dotted red arrowheads. Severely small pectoral fins with irregular edges are indicated by solid black arrowheads. Slightly curved tail is indicated by a dotted white arrow. Curved body shape is indicated by a solid white arrow. Hpe, hours post-exposure; INH, isoniazide.

malformations. However, spinal curvature, the most pronounced morphological alteration, was only observed in the 16 mM INH-treated group (Fig. 3A–D).

At 72 hpe, the 2 mM INH-treated group also demonstrated individual deformities. In the 16 mM INH-treated group, serious malformations including curved body shape, swimming bladder absence, yolk retention and tail malformation were observed (Fig. 3A–D). The group treated with 16 mM INH had a 100% malformation rate by 72 hpe (Fig. 2C). Thus, INH caused a dose- and time-dependent increase in developmental toxicity.

Swimming behavior. We performed the behavioral tests on larvae at 24, 48 and 72 hpe. The digital tracks are shown in Fig. 4(A). In the digital tracks map, the red lines are associated with fast movement; green lines are associated with medium movement; and black lines indicate slow movement. The larvae from the groups exposed to 6, 8 or 16 mM INH showed remarkable decreases in the average speed (Fig. 4B,D,F). No significant differences in the average speeds were found between the unexposed group and those exposed to 1 or 2 mM INH (data not shown). The total distance covered by the zebrafish was also significantly reduced in those treated with 6, 8 or 16 mM INH (Fig. 4C,E,G). These results revealed that INH resulted in a reduced locomotor capacity of the zebrafish larvae.

Histological analysis. The livers and intestines of the INH-treated zebrafish exhibited histopathological changes compared to the control (Fig. 5A,B). Loose cell-to-cell contacts and large vacuoles were observed in the hepatocytes of the larvae exposed to INH at doses of 8 or 16 mM at 24 hpe, 6, 8 or 16 mM at 48 hpe, and 4, 6, 8 or 16 mM at 72 hpe. Thin intestinal walls, frayed gut villi and widespread cell lysis were observed at 24, 48 and 72 hpe in the intestines in the groups exposed to the higher concentration (8, 16 mM).

Reactive oxygen species measurement. A dose- and time-dependent increase in the generation of ROS was observed when the zebrafish larvae were treated with INH (Fig. 6). The larvae in control group generated clear images whereas the larvae in the 2, 4, 6, 8 and 16 mM INH groups generated fluorescent images, which suggested that the generation of ROS had taken place in the presence of INH in the zebrafish larvae. However, no apparent changes were observed in the larvae treated with 1 mM INH.

Oxidative stress. The effects of INH on SOD and MDA are shown in Fig. 7. The SOD activity was significantly lower in the 16 mM INH-treated larvae at 24 hpe ($P < 0.05$). By 48 and 72 hpe, the SOD activities in zebrafish larvae treated with 6, 8 or 16 mM INH were significantly reduced ($P < 0.01$). The MDA levels were significantly higher in the 6, 8 or 16 mM INH-treated larvae at 24 and 48 hpe ($P < 0.05$, $P < 0.01$). By 72 hpe, the MDA levels in the zebrafish larvae treated with 4, 6, 8 or 16 mM INH were significantly increased ($P < 0.05$, $P < 0.01$).

Gene expression. To investigate the possible mechanisms of the toxic effects induced by INH exposure, we used quantitative polymerase chain reaction to examine the expression of SOD1, GSTP2, liver fatty acid-binding protein (L-FABP) and cytochrome P450 3A (CYP3A) in the total RNA isolated from the 24, 48 and 72 hpe larvae. The expression of SOD1 and L-FABP was slightly decreased in the larvae exposed to 2 mM INH. In those exposed to 6, 8 or 16 mM INH, the expression levels of SOD1 and L-FABP were significantly decreased (Fig. 8A,C). INH-induced upregulation of GSTP2 and CYP3A by 72 hpe following exposure to 6, 8 or 16 mM (Fig. 8B,D).

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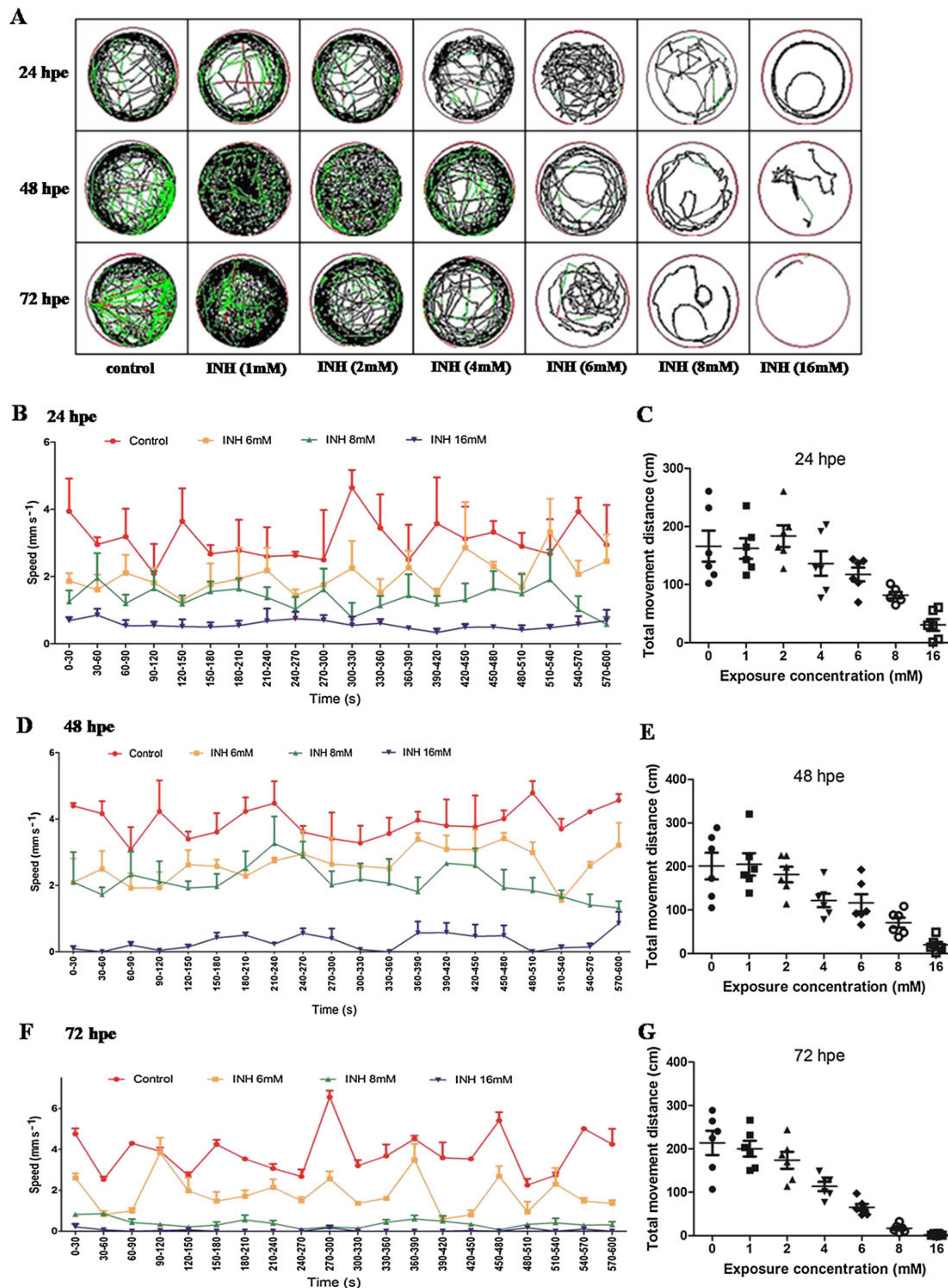

Figure 4. Reduction of locomotor capacity in INH-exposed zebrafish larvae at 24, 48 and 72 hpe. (A) Digital tracks of larvae exposed to various INH doses at 24, 48 and 72 hpe. (B,D,F) The average speed of the larvae exposed to 6, 8 or 16 mM INH at 24, 48 and 72 hpe. (C,E,G) Statistical analyses on the total movement distance of larvae exposed to 1, 2, 4, 6, 8 or 16 mM INH at 24, 48 and 72 hpe. Hpe, hours post-exposure; INH, isoniazide.

Discussion

In vitro techniques such as cell culture systems are often used for toxicity evaluations because they are both cost- and time-efficient (Busquet *et al.*, 2008). However, it is difficult to translate directly these results to effects on whole organisms and human health. *In vivo* studies require extensive facilities and infrastructure to investigate the biological responses (Henken *et al.*, 2004). Zebrafish provide many practical advantages as a powerful *in vivo* model organism for the elucidation of developmental toxicity (Shi *et al.*, 2008). Zebrafish are an outstanding model to assess the mechanisms of specific biological responses (Nishimura *et al.*, 2016).

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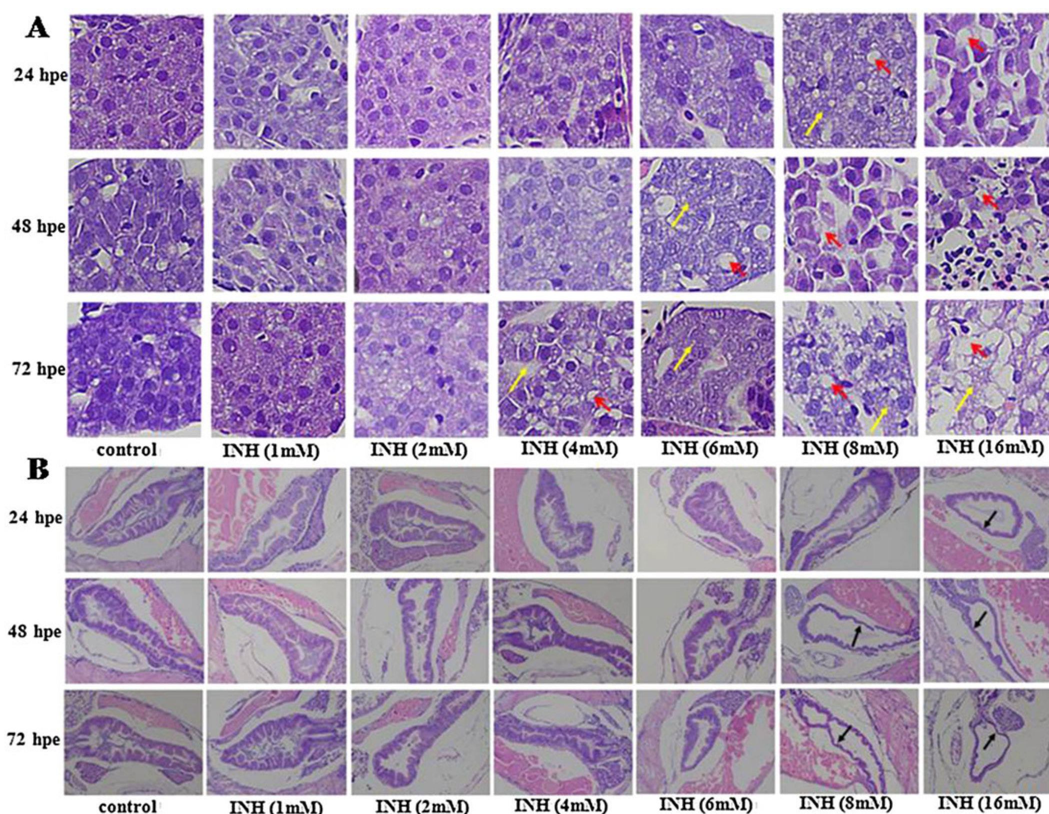

Figure 5. Histopathological changes in (A) livers and (B) intestines in zebrafish larvae exposed to INH at 24, 48 and 72 hpe. Loose cell-to-cell contacts are indicated by yellow arrowheads. Large vacuoles are indicated by red arrowheads. Thin intestinal walls and frayed gut villi are indicated by black arrowheads. Hpe, hours post-exposure; INH, isoniazide.

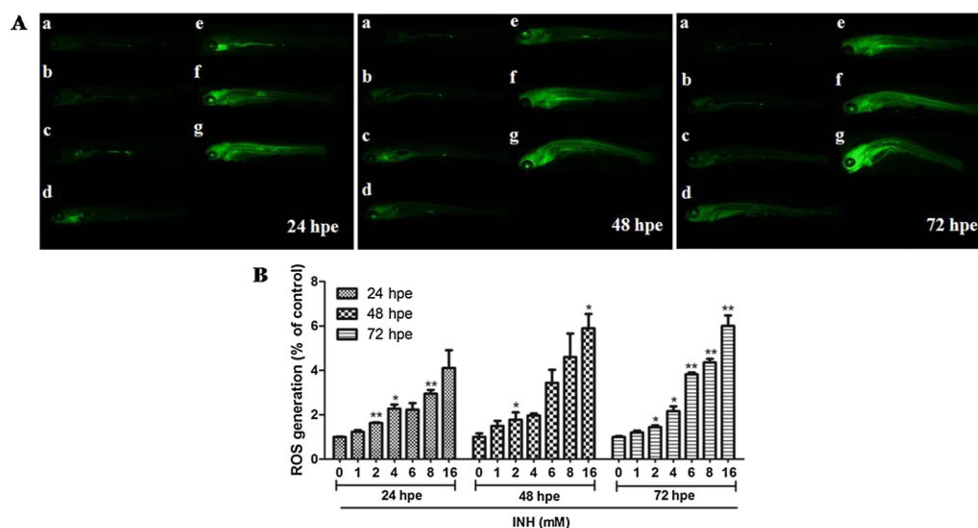

Figure 6. Effects of INH on ROS generation in zebrafish larvae. (A) Fluorescence micrographs of ROS generation in zebrafish larvae treated with: (a) control; (b) 1 mM INH; (c) 2 mM INH; (d) 4 mM INH; (e) 6 mM INH; (f) 8 mM INH; and (g) 16 mM INH. (B) Quantitative analysis of ROS generation. $*P < 0.05$, $**P < 0.01$ versus control. Hpe, hours post-exposure; INH, isoniazide; ROS, reactive oxygen species.

A selection of INH concentrations in this study referenced to the previous studies (Ahadpour *et al.*, 2015). In clinic, the dose of INH used for patients in the treatment of TB is reported as 300 mg day^{-1} (Bidarimath *et al.*, 2016). According to reports in the literature, after oral administration of INH at a dosage of 5 mg kg^{-1} in a human patient, the peak serum concentration of 10–40 μM was achieved (Bhandari & Kaur, 2013; Wang *et al.*, 2016). The 1, 2, 4, 6, 8, 16, 32 and 64 mM doses chosen for the toxicity studies in zebrafish model exceeded the clinical blood concentration (such as 40 μM) by 25-, 50-, 100-, 150-, 200- , 400-, 800- and 1600-fold,

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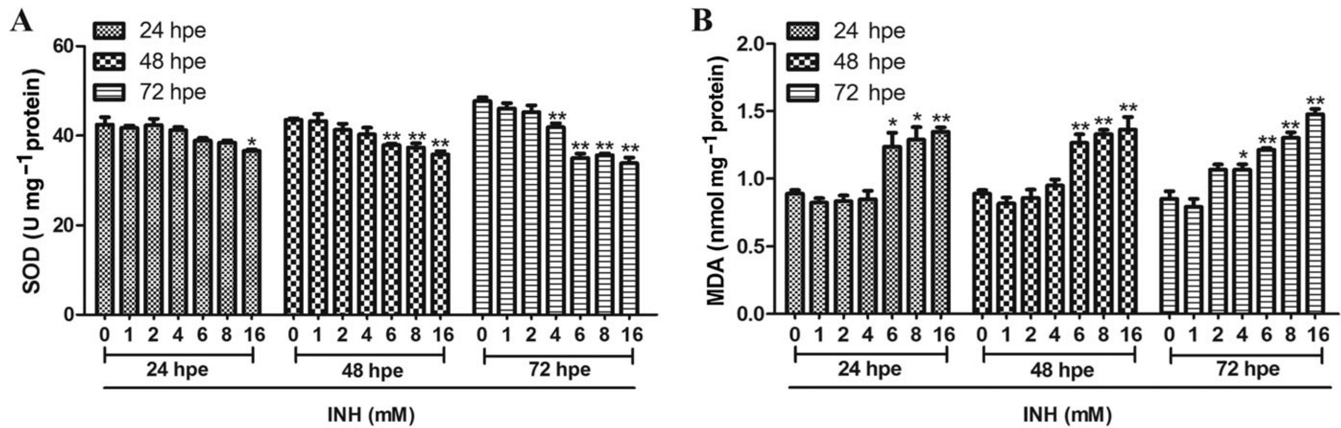

Figure 7. Changes in the activities of (A) SOD and levels of (B) MDA in INH-exposed zebrafish larvae at 24, 48 and 72 hpe. *P < 0.05, **P < 0.01 versus control. Hpe, hours post-exposure; INH, isoniazide; MDA, malondialdehyde; SOD, superoxide dismutase.

Figure 8. Gene expression of (A) SOD1, (B) L-FABP, (C) GSTP2 and (D) CYP3A following INH exposure at 24, 48 and 72 hpe ($n = 30$). *$P < 0.05$, **$P < 0.01$ versus control. CYP3A, cytochrome P450 3A; hpe, hours post-exposure; INH, isoniazide; L-FABP, liver fatty acid-binding protein; CYP3A, cytochrome P450 3A; SOD1, superoxide dismutase 1.

respectively. It is proven that exposure of experimental animals or *in vitro* models of drugs and chemicals in high doses are a necessary and valid method of discovering possible hazards in humans (Ahadpour *et al.*, 2015). Before formal experiments, a series of drug doses less than 1 mM (such as 0.1 and 0.01 mM), which are close to and below the clinical blood concentration were selected to investigate the developmental toxicity of INH. However, there were no toxic effects on the development of zebrafish embryos and larvae in the 0.01, 0.1 and 1 mM INH groups. Therefore, the doses of INH in our study ranged from no effect on the development to clear the toxic effect on development. Neither toxic effect nor adverse reaction was observed for the zebrafish embryos and

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larvae exposed to 1 mM INH-treated group. In addition, no obvious abnormalities were observed in the 2 mM INH-treated group except for decreased body length and increased ROS level. Exposure to high doses (> 6 mM) of INH significantly suppressed the embryonic development and disturbed the behaviors of the zebrafish embryos and larvae.

Our results showed that INH significantly affected the development of zebrafish embryos and larvae. This study documented time- and dose-dependent responses of zebrafish embryos and larvae to INH. In this study, a significant increase in the mortality of zebrafish embryos was observed at 72 hpe following exposure to 64 mM INH. However, for zebrafish larvae, the exposure concentration of 16 mM INH caused a significant increase in the mortality at 72 hpe. The results indicate that the zebrafish larvae were more sensitive than the embryos to the toxicity of INH. Exposure to INH has significant adverse effects on the hatching of the treated embryos. The low hatching rate is one of the critical sublethal effects of the toxicant. Delayed hatching can be attributed to delays in the zebrafish embryogenesis (Shaw *et al.*, 2016). The hatching rate for the INH-exposed embryos was decreased significantly in a dose-dependent manner compared to the control zebrafish embryos. It has been reported that delayed hatching may be due to abnormal breaks in the chorion (Osman *et al.*, 2007). This effect may also be due to the inhibition of Tetraspanin cd63 gene, which results in a deficiency in the secreted proteolytic enzymes that are required to soften the chorion (Trikic *et al.*, 2011). The growth inhibition in zebrafish embryos might be an effect of INH-mediated toxicity, which may have caused malabsorption of the nutrients required for normal development (Du *et al.*, 2012).

The developmental abnormalities observed in the embryos and larvae exposed to INH included spinal curvature, yolk retention, swimming bladder absence, tail bending and shorter body lengths. These abnormal phenotypes became more severe at higher concentrations of INH. Spinal curvature could be directly observed in the malformed embryos/larvae that had been exposed to INH. Differential accumulation of the toxicant, inhibition of acetylcholinesterase activity and a lack of neuromuscular coordination could result in spinal curvature (Pamanji *et al.*, 2015). This effect may also be due to decreasing amounts of collagen in the spinal column or changes in its amino acid composition or due the inhibitory regulation of pkt7 gene, which is a critical regulator of Wnt signaling (Hayes *et al.*, 2014). Decreased body length is an important indicator of embryo growth that is induced by the loss of nutrients. In the control group, the larvae could absorb nutrients from the yolk sac and exhibited healthy growth. The zebrafish embryos exposed to some concentrations (6, 8 and 16 mM) of INH showed a significant reduction in the inflation of their swim bladders and delayed yolk sac absorption. The yolk sac plays an important role during the early developmental stage. The yolk sac is the only source of nutrition for zebrafish embryos, and it influences the embryonic development (Qiang *et al.*, 2016). The swim bladder is an air-filled sac located dorsally in the abdominal cavity. It helps the fish to balance hydrostatic pressure and reduces the energetic cost of swimming (Johnson *et al.*, 2007). There were no deformities in the control group, but both delayed yolk sac absorption and uninflated swim bladders were observed in the INH-treated groups. These results demonstrate that INH can significantly delay the development of zebrafish embryos/larvae. The histopathological analysis showed that INH led to loose cell-to-cell contacts and large vacuoles in the hepatocytes, thinning of the intestinal walls, frayed gut villi and widespread cell lysis in the larval intestines.

Behavior is an important endpoint for the evaluation of the developmental toxicology of drugs. Significant dose- and time-dependent decreases in the total distances moved and average movement speed were observed in the INH-treated zebrafish larvae compared with the controls. This is the first report to demonstrate that INH exposure at high doses can reduce the locomotion ability. The altered larval swimming behaviors might result from the delayed neuromuscular system development (Sun *et al.*, 2016).

INH significantly increased the levels of ROS and MDA, and decreased the SOD activity in the zebrafish larvae, which suggested an induction of oxidative stress and inhibition of antioxidant capacity. Our results also indicated that INH significantly decreased the SOD1 mRNA levels, which is an effect associated with oxidative damage. Several lines of evidence suggest that a high ROS level can induce inflammation and lead to oxidative stress, which can result in various biochemical and physiological lesions (Wijesinghe *et al.*, 2013). SOD is an important antioxidant enzyme that can protect the body against oxidative damage (Jin *et al.*, 2015). MDA is a major oxidation product that has been used to monitor and assess the oxidative damage due to exposure to drugs (Hou *et al.*, 2015). The results of this study demonstrate that oxidative stress-mediated developmental toxicity was induced by INH in the zebrafish larvae.

In recent years, the activities of metabolic enzymes have been used as important biomarkers with which to assess the toxicity of xenobiotics (Jeon *et al.*, 2016). CYP3A is a member of the cytochrome P450 isoenzyme family, which is responsible for the phase I detoxification of exogenous chemicals (Creusot *et al.*, 2015). GSTP2 is a member of the GST Pi class, which is responsible for phase II cellular detoxification and plays an important antioxidant role by eliminating ROS as well through conjugation reactions with glutathione (Dong *et al.*, 2013). Many xenobiotics can modulate drug-metabolizing enzymes, and slight imbalances in the metabolic enzymes can have important chemical consequences that lead to oxidative stress or glutathione depletion (Zhang *et al.*, 2013). Therefore, the upregulation of CYP3A and GSTP2 in the zebrafish larvae observed in this study might be due to exposure to INH.

The L-FABP is an effective endogenous antioxidant that acts during the process of oxidative stress. Several observations have indicated that L-FABP plays a role in the susceptibility to ROS (Wang *et al.*, 2005). Clofibrate, an agonist of L-FABP, can increase the intracellular L-FABP levels and alleviate the oxidative stress induced by H_2O_2 incubation or hypoxia–reoxygenation (Rajaraman *et al.*, 2007). The mechanism of L-FABP's antioxidant activity probably involves inactivation of the free radicals by L-FABP's methionine and cysteine amino acids (Yan *et al.*, 2009). In this study, we observed that a decrease in the L-FABP mRNA level was associated with a statistically significant increase in the levels of ROS products and the presence of oxidative stress. These results suggested that L-FABP is a potential pharmacological target for understanding the oxidative stress-mediated developmental toxicity induced by INH.

In conclusion, INH caused a dose- and time-dependent increase in developmental toxicity. Exposure to high doses (> 6 mM) of INH significantly suppressed the embryonic development and disturbed the behaviors of the zebrafish embryos and larvae. The developmental abnormalities observed in the INH-exposed embryos and larvae included spinal curvature, yolk retention, absence of swimming bladders, bent tails and shorter body lengths. The gene expression studies showed that the aberrant expression of genes with antioxidant effects might play an important role in the INH-

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induced developmental toxicity in zebrafish larvae. Taken together, the results of this study demonstrated that oxidative stress played an important role in the developmental toxicity induced by INH in zebrafish larvae. These results suggest that the occurrence of adverse reactions should be closely monitored in pregnant women and newborns that use INH. Further study is still required to determine the impacts of INH on adult zebrafish and the next generation of the larvae. This report is a first step towards this evaluation.

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Conflict of interest

The authors did not report any conflict of interest.

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