Zebrafish model for assessing induced organ toxicity by Strychnos nux-vomica

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Abstract

OBJECTIVE: To assess the acute organ toxicity of Strychnos nux-vomica with zebrafish model visually.

METHODS: To assess acute toxicity, we initially determined the lethal concentration after Strychnos nux-vomica treatment for 24 h. Zebrafish was treated with five concentrations \( \leq \) LC10 for 24 h, and the effects of Strychnos nux-vomica on morphology, function of heart, central nervous system, liver, kidney and organ-specific cell death were assessed. Next, we assessed the reversibility of toxic effect.

RESULTS: Strychnos nux-vomica has an effect on the different organs of zebrafish, including heart, central nervous system, liver, and kidney, and cardio-toxicity induced by Strychnos nux-vomica was reversible to some extent.

CONCLUSION: Zebrafish model is suitable for confirming the toxic target organs for Chinese traditional medicine.

Key words: Zebrafish; Strychnos nux-vomica; Toxicity

INTRODUCTION

Strychnos nux-vomica, in the family Loganiaceae, is dry mature seeds of Strychnos nux-vomica Linn. It has a long history as medicine and is widely used to treat various diseases, including rheumatoid arthritis and arthralgia. The Chinese Pharmacopoeia 2010 Edition records that it is warm, bitter and poisonous, distributed to liver and spleen channels. It has the effect of treating swelling, inflammation, exciting central nervous system, and alleviating pain. However, its therapeutic use is often hindered by problems such as its strong toxicity. Therefore, it is important to strengthen the research on the compatibility of Strychnos nux-vomica, explore its effective compatibility, decrease its toxicity and increase its efficacy.

Recently, it has widely proved that zebrafish is an inexpensive and alterative model for the rapid evaluation of the potential toxicity for drug candidates since it shares the physiological, morphological, and histological similarities with mammals and human. The gene homology between zebrafish and humans is as high as 85%. In signal transduction pathways and at the protein level,
the functional domains are highly conserved or structurally identical. Compared with the traditional in vivo model like mouse and in vitro cell culture model, transparent zebrafish embryos offer unique advantages for assessing the drug effects on various developmental events because multiple organs can be observed under the microscope. As a consequence, pharmacodynamic, pharmacokinetic, and metabolite activities can be evaluated easily. In addition, low cost, short cycle, fewer test compounds, and high throughput also make it a promise and successfully used model in studying developmental toxicity, teratogenicity, cardiovascular toxicity, liver toxicity, behavioral toxicity, kidney toxicity, and a series of evaluation assays. In this study, the acute toxicities and organ toxicity induced by Strychnos nux-vomica was assessed in zebrafish. The zebrafish model was found to be an alternative or even better predictive toxicity model compared with conventional system in Strychnos nux-vomica safety assessment.

METHODS AND MATERIALS

Zebrafish breeding and handling
Embryos were generated by natural pairwise mating as described in The Zebrafish Book (Westerfield, 1993). Embryos were maintained at 28.5 °C in fish water (0.137 mol/L NaCl, 5.4 mmol/L KCl, 0.25 mmol/L NaHPO₄, 0.44 mmol/L KHPO₄, 1.3 mmol/L CaCl₂, 1.0 mmol/L MgSO₄, and 4.2 mmol/L NaHCO₃ (pH 7.0-7.2, conductivity 500-750 μs/cm)). Embryos were cleaned (dead and unfertilized embryos removed) and staged at 4 h post fertilization (hpf). Because embryos received nourishment from an attached yolk sac, no feeding was required for 7 days post fertilization (dpf).

Plant material and extraction
Strychnos nux-vomica was supplied blinded by the Anguo Shengshan Pharmaceutical Co., Ltd., (Anguo, China). Smashed into powders of 25.4 mm, the Strychnos nux-vomica (50 g) was extracted with distilled water ten-times volume of weight by refluxing for two h. After the crude extract was filtered, the supernatant was collected for further procedure, and the residue was successively extracted with distilled water ten-times volume of weight by refluxing twice, once an hour. The water extract was mixed, centrifuged (3000 × g) for 30 min (RJ-LDL-50G, Wuxi Ruijiang Analysis Instrument Co., Ltd., Wuxi, China) and the supernatant was concentrated under reduced pressure using a vacuum rotary evaporator. After that, the water separation was collected and dried under vacuum at room temperature. The dried extract was kept in a desiccator until it was used. Prior to the following biological assays, the whole extract was re-dissolved into the stock solution (400 μg/mL) with fisher water, and then diluted to the working concentrations.

Drug treatment
Different organs of zebrafish form at different stages. To ensure comparability for assessing drug-induced lethality and acute toxicity, central nervous system (CNS) and heart were assessed after treating 2 dpf zebrafish for 24 h, and liver and kidney were assessed after treating 4 dpf zebrafish for 24 h. For each concentration, 20 zebrafish were distributed into 24-well plates containing 2 mL sterile fish water, each well two larva fish. During early embryogenesis, a protective chorion membrane, which might interfere with compound uptake, was present. Therefore, to facilitate drug delivery, 2- dpf zebrafish were de-chlorinated using a 0.5 mg/mL of protease solution (P6911, Sigma-Aldrich Co., St. Louis, MO, USA) for 15 min at room temperature. 2- dpf or 4- dpf zebrafish were then incubated with compounds for 24 h. For each experiment, untreated zebrafish were used as assay control. If > 10% of embryos in the untreated control group were dead or malformed, the experiment was considered invalid and aborted.

Lethality assessment
To assess lethality, 10 concentrations were initially tested: 0.01, 0.5, 0.1, 0.5, 1, 5, 10, 50, 100, and 400 μg/mL. Based on the preliminary experiment, we set up six concentrations at last. To establish lethality curves, after treatment for 24 h, surviving zebrafish exposed to different concentrations were counted, and dead embryos were removed since dead zebrafish can disintegrate, impeding counting. Experiments were performed three times. LC₅₀ and LC₁₀ were calculated using logistic regression analysis (SPSS Statistics for Windows, Version 17.0., SPSS Inc., Chicago, IL, USA).

Assessment of organ morphology / function toxicity
Six concentrations ≤ LC₁₀, determined in lethality studies, were used to assess organ-specific toxicity. Drug treatment was performed as described above. After heart rate and circulation were assessed, zebrafish were anesthetized with 0.5 mm M3-aminobenzoic acid ethyl ester (MESAB-22, Sigma-Aldrich Co., St. Louis, MO, USA) and visually assessed using a TE-2000-S-stereomicroscope (Nikon Chansn Instrument Co., Ltd., Beijing, China) equipped with a SPOT Insight digital camera. Three percent of methylcellulose (Sigma-Aldrich Co., St. Louis, MO, USA) was used to immobilize zebrafish to facilitate imaging. For different organs, the end points for organ morphology or function toxicity are different. Abnormal heart rate, tachycardia, arrhythmia, abnormal circulation, pericardial edema and abnormal heart chamber morphology were used for evaluation standard of cardiac toxicity, misshapen brain was for CNS toxicity, liver size and color was for hepatotoxicity, and fluid accumulation around the kidney, cyst formation, or trunk edema was for renal toxicity.
Assessment of cell death after 24 h treatment
The same compound treatment strategy described in section above was used for the cell death assays. At the end point of the experiment, after washed by phosphate buffer saline (PBS) twice to remove excess drug, zebrafish samples were stained with 1 μg/mL of acri-dine orange (A8120, Sigma-Aldrich Co., St. Louis, MO, USA) in the dark environment at 28.5 °C for 0.5 h, followed which, the zebrafish, washed with PBS solution twice, were visually assessed by using a fluorescence microscope.

Assessment of reversibility of morphology / function organ toxicity
To determine if the Strychnos nux-vomica-induced acute toxicity was reversible, after treatment for 24 h, the Strychnos nux-vomica was removed by washing and the zebrafish were incubated in fresh fish water for an additional 48 h. Finally, organ toxicity was reassessed.

RESULT
LC10 and LC50 determination
Two-dpf and four-dpf zebrafish were initially treated for 24 h with different Strychnos nux-vomica concentrations ranging from 0.01 μg/mL to 400 μg/mL. Triplicate experiments were performed using 20 zebrafish per concentrations. On the basis of the results of preliminary experiment, no lethality was detected at concentrations of 10 μg/mL, and 400 μg/mL could lead all 2-dpf zebrafish to die, while the death was rare in the condition of 100 μg/mL. The additional concentrations, including 50, 100, 150, 200, 250, 300, 350, and 400 μg/mL, were then assessed in two experiments. For the 4-dpf zebrafish, 100% lethality was detected at concentration of 100 μg/mL, and no lethality was detected up to concentration of 5 μg/mL. Finally six experimental concentrations for the LC10 and LC50 determination was determined for cardiac toxicity and CNS toxicity of 2-dpf zebrafish, including 5, 10, 50, 100, 150, and 200 μg/mL, while 5, 10, 25, 50, 75, and 100 μg/mL for hepatotoxicity and renal toxicity of 4-dpf zebrafish. 2dpf zebrafish lethality curves are presented in Figure 1A and 4dpf zebrafish in Figure 1B. Based on the zebrafish lethality curves of the acute toxicity assays, LC10 of Strychnos nux-vomica for 2-dpf or 4-dpf zebrafish was calculated, and the results are 22.2 μg/mL and 7.2 μg/mL, respectively. To assess the organ toxicity, five concentrations ≤ LC10 selected for heart and CNS were 1, 5, 10, 15, 20 μg/mL, for liver and kidney 1, 2, 3, 4, 5 μg/mL, respectively.

Organ toxicity after treatment for 24 h
Organ morphology and function were assessed after Strychnos nux-vomica treatment for 24 h at 2-dpf or 4-dpf stage. For each experiment, in addition to compound-treated zebrafish, untreated zebrafish were assessed concurrently. Results for assessing toxic effects on heart rate were recorded at four h post-treatment and 8 h post-treatment, and were analyzed for statistical significance (Table 1). All data between 4 h and 8 h post-treatment were analyzed by paired-sample to compare the comprehensive evaluation values, and the re-

![Graph A: Strychnos nux-vomica induced zebrafish death rate at 2 dpf or 4 dpf stage](image1)

![Graph B: Strychnos nux-vomica induced zebrafish death rate at 2 dpf or 4 dpf stage](image2)

**Figure 1** Strychnos nux-vomica induced zebrafish death rate at 2 dpf or 4 dpf stage
A: 2dpf zebrafish mortality rate; B: 4dpf zebrafish mortality.
results showed that there was a significant difference in heart rate in 10 s of zebrafish embryos between before Strychnos nux-vomica treatment and after treatment with the concentration ≥ 50 μg/mL (P < 0.01). The results proved that the heart rate was significantly decreased by Strychnos nux-vomica exposure in a dose-dependent manner. At the five concentrations ≤ LC10, there was no significant difference in effect on heart rate between Strychnos nux-vomica treatment group and the control group. Results showed that Strychnos nux-vomica caused toxicity in all four organs: heart, CNS, liver, and kidney. Except reduced heart rate, pericardial edema in heart was also observed. Tissue discoloration was observed in the brain, indicating there was degeneration (Figure 2). Discolored liver and retained yolk was observed (Figure 3). Trunk edema indicated potential defective kidney function was observed at the high concentrations but not at the concentrations ≤ LC10.

Cell death assessed after 24 h treatment
After using the same treatment conditions for visually assessing organ toxicity, we assessed the cell death. Two or four-dpf zebrafish were treated with Strychnos nux-vomica for 24 h, and cell death in heart, CNS, liver, and kidney was assessed. Compound-induced cell death in each organ was assessed visually by staining with fluorescent acridine orange, and the results are shown. After exposed to Strychnos nux-vomica, cell death was observed in heart and CNS (Figure 4), as well as liver and kidney (Figure 5). Compared with the control group that there were only a few apoptotic cells existing, the number of apoptotic cells in the heart, CNS, kidney and liver in the treated group increased in a concentration-dependent manner.

Evaluation of reversibility of toxicity 48 h after strychnos nux-vomica removal
In order to evaluate if Strychnos nux-vomica-induced acute toxicity was reversible, after treatment for 24 h, zebrafish were washed in fresh fish water to remove Strychnos nux-vomica and incubated for an additional 48 h, and organ morphology and function were then reassessed visually in the surviving animals. Although heart rate in the treatment group at the five concentrations ≤ LC10 was not significantly different with that in the control group, the presented pericardium edema was significantly improved (Figure 6). Besides, the heart rate of surviving animals at the concentrations of 50 and 25 μg/mL was also significantly improved. For the liver and kidney, while discolored liver, retained yolk, trunk edema was significantly improved (Figure 7), but it still existed, and the cell death assessment showed that cell death was observed in liver and kidney. These results indicate that the heart toxicity induced by Strychnos nux-vomica is reversible.

DISCUSSION
Most zebrafish assays rely on aqueous exposure and its size, low cost, fecundity, rapid development of most organs within 48 hpf, capacity to absorb low molecular weight compounds which dissolved in the culture water through their chorion, skin, and gills, making it suit for small molecule screening, especially for...
Figure 3 Effect of Strychnos nux-vomica on morphology of liver and kidney of zebrafish at 5-dpf (×10)
4 dpf zebrafish were treated with different concentrations Strychnos nux-vomica for 24 h. A: normal group; B: Strychnos nux-vomica 1 μg/mL group; C: Strychnos nux-vomica 5 μg/mL group; D: Strychnos nux-vomica 10 μg/mL group; E: Strychnos nux-vomica 15 μg/mL group; F: Strychnos nux-vomica 20 μg/mL group. Arrows indicate retained yolk, the size of which is increased in a dose-dependent manner. Strychnos nux-vomica caused liver toxicity and kidney toxicity in a dose-dependent manner.

Figure 4 Cell death assessed in heart and central nervous system in 3-dpf zebrafish at the concentrations (×10)
3 dpf zebrafish treated with different concentrations Strychnos nux-vomica for 24 h were stained by acridine orange. A: normal group; B: Strychnos nux-vomica 1 μg/mL group; C: Strychnos nux-vomica 5 μg/mL group; D: Strychnos nux-vomica 10 μg/mL group; E: Strychnos nux-vomica 15 μg/mL group; F: Strychnos nux-vomica 20 μg/mL group. Autofluorescence, particularly in the heart and CNS, was observed during exposure fluorescence light. White arrows show cell death in the heart and red arrows show cell death in the CNS. With the concentration increased, the density and area of fluorescent in heart and CNS is increased. CNS: central nervous system.
Figure 5 Cell death assessed in kidney and liver in 5-dpf zebrafish at the concentrations (× 10)
5 dpf zebrafish treated with different concentrations Strychnos nux-vomica for 24 h were stained by acridine orange. A: normal group; B: Strychnos nux-vomica 1 μg/mL group; C: Strychnos nux-vomica 5 μg/mL group; D: Strychnos nux-vomica 10 μg/mL group; E: Strychnos nux-vomica 15 μg/mL group; F: Strychnos nux-vomica 20 μg/mL group. Autofluorescence, particularly in the liver and kidney, was observed during exposure fluorescence light. White arrows show cell death in the liver and red arrows show cell death in the kidney. With the concentration increased, the density and area of fluorescent in liver and kidney is increased.

Figure 6 Reversibility of toxicity assessed in heart and central nervous system in 5-dpf zebrafish at the different concentrations (× 10)
3 dpf zebrafish treated with different concentrations Strychnos nux-vomica for 24 h were cultured in the fresh water for 48 h. A: normal group; B: Strychnos nux-vomica 1 μg/mL group; C: Strychnos nux-vomica 5 μg/mL group; D: Strychnos nux-vomica 10 μg/mL group; E: Strychnos nux-vomica 15 μg/mL group; F: Strychnos nux-vomica 20 μg/mL group. White arrows show reversibility in the heart and red arrows showed reversibility in the CNS. 48 h after removing, the pericardium edema in heart and misshapen brain with dark brown was significantly improved in a dose-dependent manner. CNS: central nervous system.
high-throughput screening since the analysis of the effects of compounds in zebrafish larvae requiring lower compound.\textsuperscript{16,17}

In the acute toxicity assays, Strychnos nux-vomica showed large variation in their acute toxicity to the heart, brain, liver and kidney, and the Strychnos nux-vomica had the lower LC\textsubscript{50} value (102.2 μg/mL) for former while Strychnos nux-vomica had the higher LC\textsubscript{50} value(47.2 μg/mL) for later. Also, acute toxicity of zebrafish also showed that zebrafish have strong sensitivity to toxicity since low concentration of Strychnos nux-vomica can cause adverse effects of the heart, kidney, liver and CNS of zebrafish. It has a good correlation with other mammalian models which belongs to the liver meridian. Also, the result showed that low concentration can cause changes in the phenotype of heart, kidney, liver and CNS of zebrafish.

In the experiment of evaluation of reversibility of toxicity, CNS and heart toxicity were observed after 24 h treatment, but those toxicity were significantly improved 48 h after Strychnos nux-vomica compound was removed, indicating the organ toxicity was reversible. Cell death was observed in liver and kidney after 24 h treatments, and cell death in these organs was still found 48 h after compound removed, which indicate that there is the delayed toxicity. Despite all of these, however, for Strychnos nux-vomica, there are still lots of problems in theory and experiment method, such as lack of a complete experimental operating standard and organism toxicity phenotypic evaluation standards, as well as sufficient data to compared results in zebrafish with results in mammalian models. The ultimate goal of toxicity experiment is to determine the concentration with which no observable toxicity in induced. In the future studies, we hope that the toxicity of Strychnos nux-vomica on any of the four organs (heart, brain, liver, or kidney) of zebrafish would be counted and expressed as percent incidence for each condition. Percent incidence and concentration would be then correlated, and no observed effect concentration would be estimated for Strychnos nux-vomica.

Certainly, the research of this thesis is only a preliminary attempt to probe the applicability of the zebrafish model in the toxicity studies of toxic Chinese traditional medicine. Furthermore, it is necessary to make full use of the advantages of the zebrafish to provide a basis for the studies of toxic Chinese medicine toxicity in the future. In conclusion, zebrafish model has become a valuable tool for assessing and predicting Traditional Chinese Medicine toxicity.

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