Original Article

Evaluation of the teratogenic effects of three traditional Chinese medicines, Si Jun Zi Tang, Liu Jun Zi Tang and Shenling Baizhu San, during zebrafish pronephros development

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Abstract: The aim of this study was to evaluate the teratogenic effects of three common Chinese medical prescriptions, Si Jun Zi Tang (SJZT), Liu Jun Zi Tang (LJZT) and Shenling Baizhu San (SLBS), during zebrafish pronephros development. We used the transgenic zebrafish line *Tg(wt1b:EGFP)* to assess the teratogenic effects using 12 different protocols, which comprised combinations of 4 doses (0, 25, 250, 1,250 ng/mL) and 3 exposure methods [methods I, 12–36 hours post fertilization (hpf), II, 24–48 hpf, and III, 24–36 hpf]. As a result, few defects in the kidneys were observed in the embryos exposed to 25 ng/mL of each medical prescription. The percentage of kidney malformation phenotypes increased as the exposure concentrations increased (25 ng/mL, 0–10%; 250 ng/mL, 0–60%; 1,250 ng/mL, 80–100%). Immunohistochemistry for α6F, which is a basolateral and renal tubular differentiation marker, revealed no obvious defective phenotypes in either SJZT- or LJZT-treated embryos, indicating that these Chinese medical prescriptions had minimal adverse effects on the pronephric duct. However, SLBS-treated embryos displayed a defective phenotype in the pronephric duct. According to these findings, we suggest (1) that the Chinese medical prescriptions induced kidney malformation phenotypes that are dose dependent and (2) that the embryonic zebrafish kidney was more sensitive to SLBS than SJZT and LJZT. (DOI: 10.1293/tox.2013-0045; J Toxicol Pathol 2015; 28: 141–149)

Key words: Chinese medical prescriptions, kidney, nephrotoxicity, zebrafish

Introduction

Many phytochemicals, nutritional supplements, micronutrients and natural medicines originated from traditional medicine bioresources. Worldwide, traditional oriental medical doctors utilize traditional Chinese medical prescriptions (CMPs) in clinical practice to remedy diseases. A CMP is a formulation that contains greater than two types of herbs and is used by clinical doctors in Eastern countries such as Hong Kong, Japan, Korea, Taiwan and China. Si Jun Zi Tang (SJZT) is one of the CMPs commonly used as an intestinal digestion regulator. Two related prescriptions of SJZT, Liu Jun Zi Tang (LJZT) and Shenling Baizhu San (SLBS), are commonly used for treating patients with poor appetite, loose stool, abdominal distension, lassitude, prolapsed anus, shortness of breath, dysphasia and spontaneous sweating[1–4]. Chinese medical doctors dispense the appropriate prescription based on syndrome differentiation. In addition, a CMP (aqueous extract) can be used for recovery from anemia and in treating digestive disease[2–4]. Recently, people like natural products and alternative medicines such as nutrient supplements for prevention of chronic disease. Notably, anti-aging and immunoregulation are the most popular goals of integrative health care with traditional CMPs. The safety of CMPs is an important issue that should be investigated.

In general, overdosing of these herbs is not recom-

mended, as it can cause a stifling sensation in the chest, abdominal distension or poor appetite (http://www.mohw.gov.tw/EN/Ministry/Index.aspx). Recently, many disease treatment-related herbal toxicities have been observed (http://www.mohw.gov.tw/EN/Ministry/Index.aspx). Aristolochic acid (AA) is commonly found in plants of the *Aristolochia* genus[5]. Clinically, the AA component of certain CMPs has been found to result in kidney failure or bladder cancer[1]. In the rat and zebrafish, AA was demonstrated to have adverse effects on the kidney, such as tubular atrophy, necrosis and lymphocytic infiltration, and consequently resulted in renal failure[6, 7]. AA-containing CMP are not allowed to be dispensed in clinics in Taiwan. But in Taiwan, Hong Kong and China, women like to employ CMPs (especially SJZT)[8] or CMP-containing food for taking good care of their health during menstruation, pregnancy, child birth and baby nursing periods. So far, the data concerning the teratogenic effects, especially those concerning developmental nephrotoxicity, are incomplete. In this study, we evaluated the teratogenic effects of the most popular CMPs (SJZT, LJZT, SLBS) during zebrafish pronephros development despite the fact that they are supposed to be AA free (containing nothing derived from plants of the *Aristolochia* genus in their ingredients).

The zebrafish is a popular animal model for toxicological studies because its embryo is completely transparent, has a clear genetic background and undergoes rapid organogenesis[9–11]. In particular, the transgenic zebrafish line *Tg(wt1b:EGFP)*, in which the glomerulus (gl), pronephric tube (pt), pronephric duct (pd) and exocrine pancreas are labeled by green fluorescent protein (GFP), is a useful tool for dynamically studying teratogenic effects during zebrafish pronephros development[11]. Many reports have demonstrated that kidney fluorescent transgenic zebrafish are efficient for studying teratogenic effects and kidney development[12–15]. In this study, we chose *Tg(wt1b:EGFP)* as a model system and performed a series of time- and dose-dependent CMP (SJZT, LJZT and SLBS)-exposure experiments. Subtle changes in kidney development were easily observed by the detection of GFP or by staining with a well-known basolateral and renal tubular differentiation marker, monoclonal antibody α6F. This strategy was excellent for studying CMP-induced teratogenic effects during zebrafish pronephros development.

Material and Methods

Ingredients of Si Jun Zi Tang, Liu Jun Zi Tang and Shenling Baizhu San

The herbs in the study herein were purchased from the crude drug market in Taipei and were processed and sliced for dispensing the following three prescriptions: (1) Si Jun Zi Tang (SJZT, 26 g/day), consisting of ginseng (*Panax ginseng*) 6 g, Atractylodes (*Atractylodes macrocephala*) 6 g, hoelen (*Poria cocos*) 6 g, licorice root preparation (*Glycyrrhiza glabra* L.) 3 g and ginger (*Zingiber officinale*) 3 g, jujube (*Zizyphus jujuba*) 2 g; (2) Liu Jun Zi Tang (LJZT, 30

g/day), consisting of ginseng (*Panax ginseng*) 5 g, Atractylodes (*Atractylodes macrocephala*) 5 g, hoelen (*Poria cocos*) 5 g, licorice root preparation (*Glycyrrhiza glabra* L.) 2.5 g, ginger (*Zingiber officinale*) 2.5 g, jujube (*Zizyphus jujuba*) 2.5 g, dried tangerine peel (*Citrus reticulata*) 2.5 g and Pinellia tuber (*Pinellia ternate*) 5 g; and (3) Shenling Baizhu San (SLBS, 22.5 g/day), consisting of ginseng (*Panax ginseng*) 3 g, Atractylodes (*Atractylodes macrocephala*) 3 g, hoelen (*Poria cocos*) 3 g, licorice root preparation (*Glycyrrhiza glabra* L.) 3 g, Chinese yam (*Dioscorea batatas*) 3 g, lotus fruit (*Nelumbo nucifera*) 1.5 g, Platycodon root (*Platycodon grandiflorum*) 1.5 g, Job's tears (*Coix lacryma-jobi* L.) 1.5 g, Amomum seed (*Amomum xanthioides* Wall.) 1.5 g, jujube (*Zizyphus jujuba*) 1.5 g (Chinese herb database; http://libproject.hkbu.edu.hk/was40/detail?channelid=35734&searchword=herb_id=D00630).

Preparation of CMP extracts

The methods for preparation of the CMPs were according to the rules of the Ministry of Health and Welfare, Republic of China (http://www.mohw.gov.tw/EN/Ministry/Index.aspx). In brief, each prescription (for humans) dispensed in a two-day dose (SJZT, 52 g; LJZT, 60 g; SLBS, 45 g) was ground in a rotary speed mill and extracted twice with 10 volumes of distilled water. The filtrates were combined, and the water was evaporated under reduced pressure at 40°C in a rotary evaporator. The three extracts (SJZT, LJZT, SLBS) were then freeze-dried and stored at −20°C. Twenty milligrams of dried extract was dissolved in phosphate-buffered solution (PBS) as the stock test sample solution (20 mg/mL). For subsequent assays, each test solution was serially diluted with PBS.

Fish embryo maintenance and staging

Mature zebrafish of the wild-type (WT; AB strain) and *Tg(wt1b:EGFP)* strains[15] were raised at the zebrafish facility of the Life Sciences Development Center, Tamkang University, and maintained at 28°C with a photoperiod of 14 hours (h) light and 10 h dark in an aquarium [30 (H)×15 (W)×20 (D) cm] supplied with fresh water and aeration[16–19]. For spawning and embryos collection, around five pairs of zebrafish were put together in another fish tank. Embryos were collected using standard procedures and staged according to standard criteria [hours post fertilization (hpf)][16]. Once enough embryos were collected, the embryos were divided into the test groups for the subsequent CMP treatment experiments.

CMP treatment

Three exposure protocols (methods I–III) were applied in the CMP treatment experiments based on combinations of different exposure onsets (12 and 24 hpf; method I vs. II) and durations (12 and 24 h; method II vs. III, Fig. 1A). For dose titration, *Tg(wt1b:EGFP)* embryos were collected, randomly divided into 90 (for experimental groups, 3 doses, 3 methods, 3 drugs, repeated 3 times, 3×3×3×3=81; for mock, 3 methods repeated 3 times, 3×3=9) groups (30 embryos per
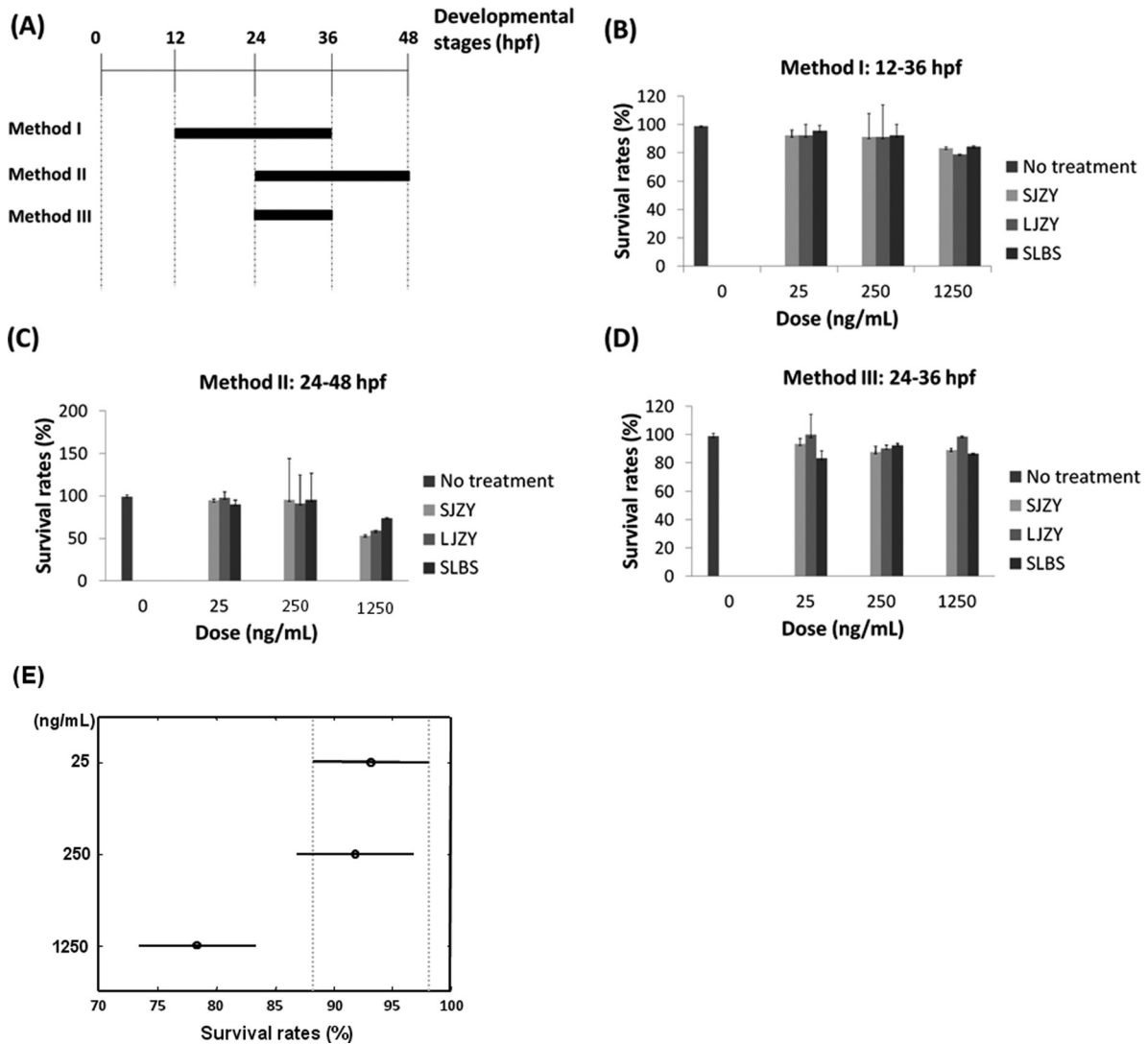
Fig. 1. (A) Exposure methods of CMP treatments used in this study. Three CMP exposure methods were designed based on exposure onset and duration: method I, 12–36 hpf; method II, 24–48 hpf; and method III, 24–36 hpf. (B, C, D) Survival rates of zebrafish embryos after exposure to water containing 25, 250; and 1,250 ng/mL of CMP (SJZT, LJZT, SLBS) using exposure methods I, II; and III. (E) The Tukey-Kramer HSD (honestly significant difference) test reported the marginal mean survival rates and corresponding 95% confidence intervals for the three dose level groups, which were adjusted for method and CMP effects. Two group means are significantly different if their intervals are disjoint; and are not significantly different if their intervals overlap.

group) and exposed to either water (mock-treated control: 0 ng/mL) or water containing the CMP at the desired concentration (25, 250 and 1250 ng/mL). All embryos were cultivated in 24-well cell culture plates, and survival rates were determined at 48 hpf. For kidney malformation recording, kidneys of CMP-treated fish at 48 hpf were compared with kidneys of healthy fish from the mock control group and subjectively classified as normal (all sites, including pt, pd and gl, are normal); or malformed kidneys (at least one site in pt, pd or gl is defective).

Histology, immunohistochemistry and images

The procedures for embedding and cryosectioning were according to those described by Pai and Chen[19] ex-cept that embryos from the mock control and CMP-treated groups (250 ng/mL) at 48 hpf were used and fixed in Dent's buffer (80% methanol, 20% DMSO) as described previous-ly[7]. Sections of 8–10 μm in thickness obtained from the pro-nephric regions were subjected to hematoxylin (H) staining using standard procedures[20–24]. The localization of the pro-nephric ducts in the cryosections was visualized by immu-nohistochemistry using a mouse monoclonal antibody (α6F, Developmental Studies Hybridoma Bank) and detected us-ing an ABC staining system (Santa Cruz) and 3,3′-diami-nobenzidine (DAB) as chromogens[25–27]. All embryos were observed under a microscope (DM 2500, Leica) equipped with Nomarski differential interference contrast optics and a fluorescent module having a GFP filter cube (Kramer Sci-

entific). Images of embryos were captured at specific stages with a CoolSNAP CCD (Photometrics).

Statistical analysis

All analyses in this study were carried out with the MATLAB software (version 7.7 R2008b). An N-way ANOVA (analysis of variance) was applied to test the effects of multiple factors (method, dose level, CMP) on the mean of the outcome variable (survival rate or malformation rate). The p-value for each factor, reported by N-way ANOVA, represents that associated with the null hypothesis that the samples at all levels of the factor are drawn from the same population. The Tukey-Kramer HSD (honestly significant difference) test was further used to compare the population marginal means for each combination of certain factors, which were adjusted by removing effects of other factors. A significance level of 0.05 was used in all statistical analyses, and a family-wise error rate of 0.05 was used in the Tukey-Kramer HSD test.

Results

Titration of CMP (SJZT, LJZT, SLBS) doses and survival rate analyses

Our results showed that $93.3 \pm 6.7\%$ to $100 \pm 0.0\%$ (mean ± standard error; SE) [n=30 (numbers of tested embryos in each group), N=3 (in triplicate experiments)] of the embryos were alive at 48 hpf after exposure to 25 and 250 ng/mL of SJZT, LJZT and SLBS (mock, 100%); however, the survival rates decreased to $53.3 \pm 8.1\%$ to $86.7 \pm 6.7\%$ (n=30, N=3) when the exposure dose was increased to 1,250 ng/mL (Figs. 1B–1D).

Statistically, three-way ANOVA was first applied to the entire dataset (methods I, II, III; doses, 25, 250, 1,250 ng/mL; CMPs, SJLT, LIZT, SLBS) to evaluate the effects of method, dose; and CMP on the survival rate. The p-values reported for the method, dose; and CMP effects were 0.1605, 0.0009, and 0.8850, respectively, indicating that there was at least one significant difference in survival rates between the three dose level groups. The Tukey-Kramer HSD test was then used to pairwise compare the marginal mean survival rates for the three dose level groups, which were adjusted for the method and CMP effects. The adjusted mean survival rates reported for the 25, 250; and 1,250 ng/mL dose level groups were 93.21%, 91.85%; and 78.40%, respectively, with a common standard error of 2.94%; these results demonstrated that the survival rates for the two low dose groups did not differ from each other but differed significantly from the high dose group (1,250 ng/mL) at a family-wise error rate of 0.05 (Fig. 1E).

Visible and defective phenotypes of embryos after exposure to CMP

Using the transgenic zebrafish line *Tg(wt1b:EGFP)*, we conducted three exposure protocols (methods I, II and III, Fig. 1A) to examine the phenotypic defects caused by differ-

ent concentrations of CMP; particular attention was focused on defects in the glomerulus, pronephric tube and pronephric duct. At 48 hpf, few phenotypic defects in the glomerulus (slightly separated), pronephric tube and pronephric duct (curved and slightly deformed) were observed in the embryos treated with 25 ng/mL CMP. However, the embryos treated with higher concentrations (250 and 1,250 ng/mL) of CMP displayed more malformed kidney phenotypes at 48 hpf. These malformed kidney phenotypes included (1) a separated glomerulus and (2) a curved pronephric tube and duct with a swollen glomerulus. An example for SJZT exposure is shown in Figure 2 (Fig. 2A–C vs. 2D, 2E).

Traditional CMP-induced kidney malformation phenotypes are dose dependent

To further study the specific roles of the CMP in kidney embryogenesis, we calculated the malformation rates at 48 hpf after CMP treatment. The results showed that few defects in the kidneys were observed in embryos treated with 25 ng/mL SJZT (Fig. 3A, 3B). As the exposure dose of SJZT increased to 1,250 ng/mL, all of the embryos displayed kidney malformation phenotypes (Fig. 3A, 3B). Moreover, when the same exposure protocols (methods I, II and III) were applied to different concentrations (0, 25, 250; and 1,250 ng/mL) of LJZT and SLBS, the percentages of kidney malformation phenotypes increased as the exposure concentrations (0 ng/mL, 0%; 25 ng/mL, 0–10%; 250 ng/mL, 0–60%; 1,250 ng/mL, 80–100%) and durations increased (method II, 10–100%; method III, 5–100%), as shown in Figs. 3B and 3C. In addition to exposure dose and duration, the exposure onset is an important point that should be discussed. In this regard, we treated zebrafish embryos with 25 ng/mL of CMPs for 24 hours with two exposure onsets (12 hpf; method I, 24 hpf; methods II) and observed the malformed kidney phenotypes at 48 hpf (Figs. 3A vs. 3B). The results showed that no embryos displayed malformed kidney phenotypes when exposure started at 12 hpf (method I) (Fig. 3A). With later exposure onsets, the proportion of embryos with malformed kidney phenotypes increased gradually (from 10% to 52.4%, Fig. 3B). Interestingly, we found that 52.4% of embryos exposed to 25 ng/mL SLBS had malformed kidneys, suggesting that SLBS is more toxic than both SJZT (35%) and LJZT (10%) at a low concentration (25 ng/mL). When the exposure concentration was increased to 1,250 ng/mL, almost all embryos displayed kidney malformation phenotypes (95%–100%, Figs. 3A–C).

The results of three-way ANOVA showed that the p-values for method, dose; and CMP effects were <0.0001, <0.0001; and 0.3661, respectively, again indicating that there was a significant difference in the malformation rates between the methods and dose level groups. The results of the Tukey-Kramer HSD test indicated that there was no significant difference in malformation rates among the CMP groups for the three methods, which were adjusted for dose effect (Fig. 3D).
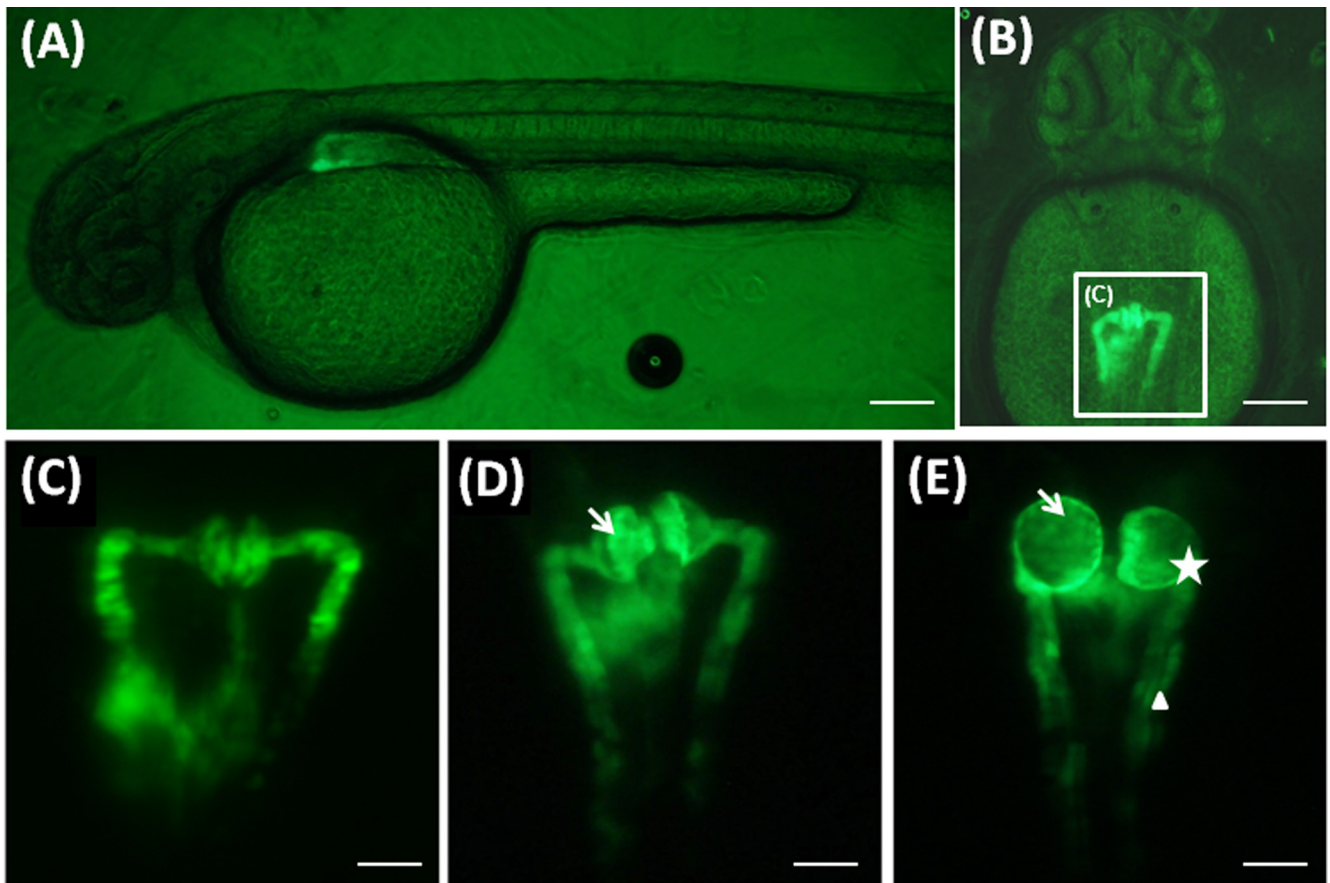
Fig. 2. Visible and defective phenotypes of *Tg(wt1b:EGFP)* embryos after exposure to SJZT (250 ng/mL). (A) Lateral and (B) dorsal view of *Tg(wt1b:EGFP)* embryos. (C) No defect. (D) Separated glomerulus. (E) Curved pronephric tube and duct and swollen glomerulus. Figures C, D and E were obtained from a dorsal view and at the developmental stage of 48 hpf. gl, glomerulus; pd, pronephric duct; pt, pronephric tube. An arrow, star and triangle indicate the defective sites in gl, pt; and pd, respectively. Scale bar: 80 μm (A, B), 40 μm (C–E).

Histopathology in the malformed kidney

We further examined the malformed kidney pheno-types at the histological level. We stained the zebrafish embryos with a monoclonal antibody (α6F) to visualize the pronephric tube and pronephric duct. After cryosection-ing followed by hematoxylin (H) and monoclonal antibody (α6F) staining, it was observed that the pronephric duct of the mock control embryo was composed of a single layer of mesenchymal cells that contributed to a compact struc-ture (Fig. 4A, 4A'). No obvious defective phenotypes were observed in either SJZT- or LJZT-treated embryos (250 ng/ml), indicating that SJZT and LJZT had no obvious adverse effects on the pronephric duct (Fig. 4B, 4C, 4B', 4C'). How-ever, the SLBS-treated (250 ng/mL) embryos displayed dis-organized and broken mesenchymal cells (Fig. 4D, 4D'). According to these findings, we suggest that the embryonic zebrafish kidney might be more sensitive to SLBS than SJZT or LJZT.

Discussion

In this study, we used a green fluorescent kidney trans-genic zebrafish to assess the CMP-induced nephrotoxicity. Transgenic zebrafish provided an alternative model that can be used to evaluate CMP-induced nephrotoxicity in a quicker, cheaper and more efficient manner. Developmental nephrotoxicity in humans is very complicated and difficult to study. In a mouse model, a large-scale preclinical study was conducted to detect the adverse effects of 20 commonly used CMPs during pregnancy, and the results showed that embryo growth retardation, fetal resorption and skeletal malformations were the evident adverse effects[28]. However, the defects in specific organs (especially the kidney) are hard to record. In this study, transgenic zebrafish provided an alternative model that can be used to evaluate CMP-in-duced nephrotoxicity in a quicker, cheaper and more effi-cient manner.

The kidney development process is conserved between mammals and zebrafish, and this makes it efficient to ac-
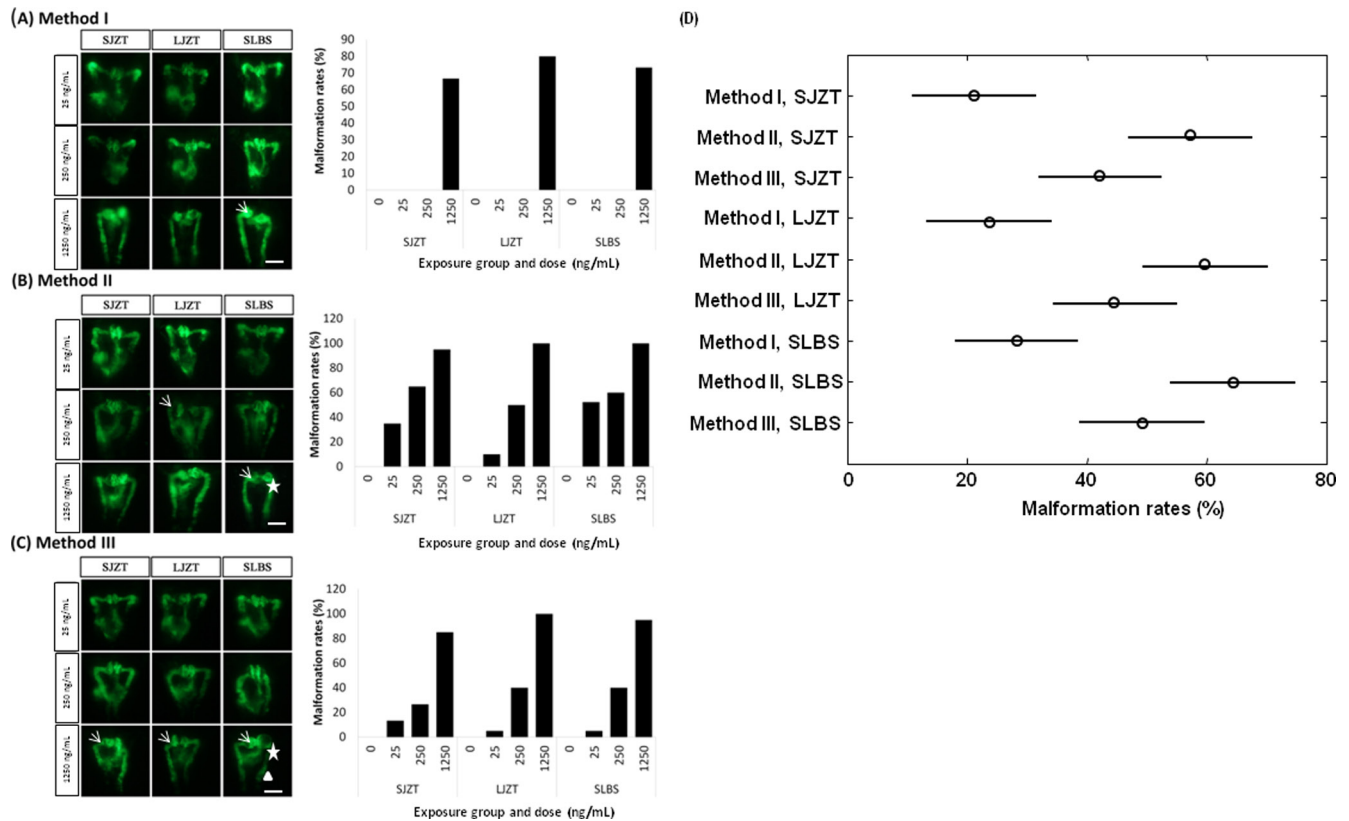
Fig. 3. CMP-induced kidney malformation phenotypes are dose dependent. Morphological changes of Tg(*wt1b*:GFP) embryos after exposure to 25, 250, and 1,250 ng/mL of CMP (SJZT, LJZT, SLBS). Phenotype percentages of zebrafish embryos after CMP treatments using exposure methods I, II, and III (A, B, C). All photos were taken from a dorsal view at the developmental stage of 48 hpf. (D) The Tukey-Kramer HSD (honestly significant difference) test reported the marginal mean malformation rates and corresponding 95% confidence intervals for combinations of the method and CMP groups, which were adjusted for dose effect. Two group means are significantly different if their intervals are disjoint; and are not significantly different if their intervals overlap. An arrow, star and triangle indicate the defective sites in gl, pt; and pd, respectively. Scale bar: 40 μm.

cess nephrotoxicity in embryonic zebrafish. Basically, the process by which the zebrafish kidney develops can be divided into four stages: (1) specification of the mesodermal cells to the nephric lineage (~12 hpf), (2) epithelialization of the duct (16–24 hpf), (3) nephron patterning (30–40 hpf), and (4) angiogenesis (40–48 hpf)[27]. According to these developmental stages, three exposure protocols were designed in this study [method I (specification and epithelialization), method II (nephron patterning and angiogenesis), method III (nephron patterning)]. Our results showed that embryos displayed less malformed kidney phenotypes when exposure started at 12 hpf (Fig. 3A vs. 3B, 3C). This suggested that the kidney developed by the nephron patterning stage might be more sensitive to toxin exposure in this study.

In a toxicological study, the exposure dose is the most important issue to be addressed. The recommended daily doses for a 60 kg human adult are 26 g for SJTZ, 30 g for LJZT and 22.5 g for SLBS. After the appropriate calculations, the doses used in this study (25–1,250 ng/mL) were approximately 1/60,000–1/12,000 of the recommended daily doses. When dividing by body weight (human, 60 kg; zebrafish embryo, 1.2 mg; adult zebrafish, 1 g), the range

of CMP doses in the zebrafish embryo treatments was approximately 16.6 to 84-fold higher than the acceptable daily intakes in adult humans. For acute toxicity testing, rapid procedures are used to measure the concentration that will affect the test animals in a single exposure or in multiple exposures in a short period of time[29]. In general, the doses of a tested substance are allowed to be higher than for the recommended daily intake[30]. Thus, we suggest that 16.6 to 84-fold is an acceptable range for assessment of the acute nephrotoxicity of a CMP in a zebrafish model.

Our data clearly showed that a curved pronephric tube and duct with a swollen glomerulus were evident in CMP-induced malformed kidney phenotypes (Fig. 2). Figure 4 shows that disorganized and broken mesenchymal cells around the pronephric ducts were also be observed in the CMP-treated embryos. It was previously reported that drug-induced glomerular permeability impairment led to accumulation of blood cells and caused kidney edema[31]. Thus, we suggest that CMP-induced malformed kidney phenotypes might be highly probably related with CMP-induced glomerular/mesenchymal permeability impairment.
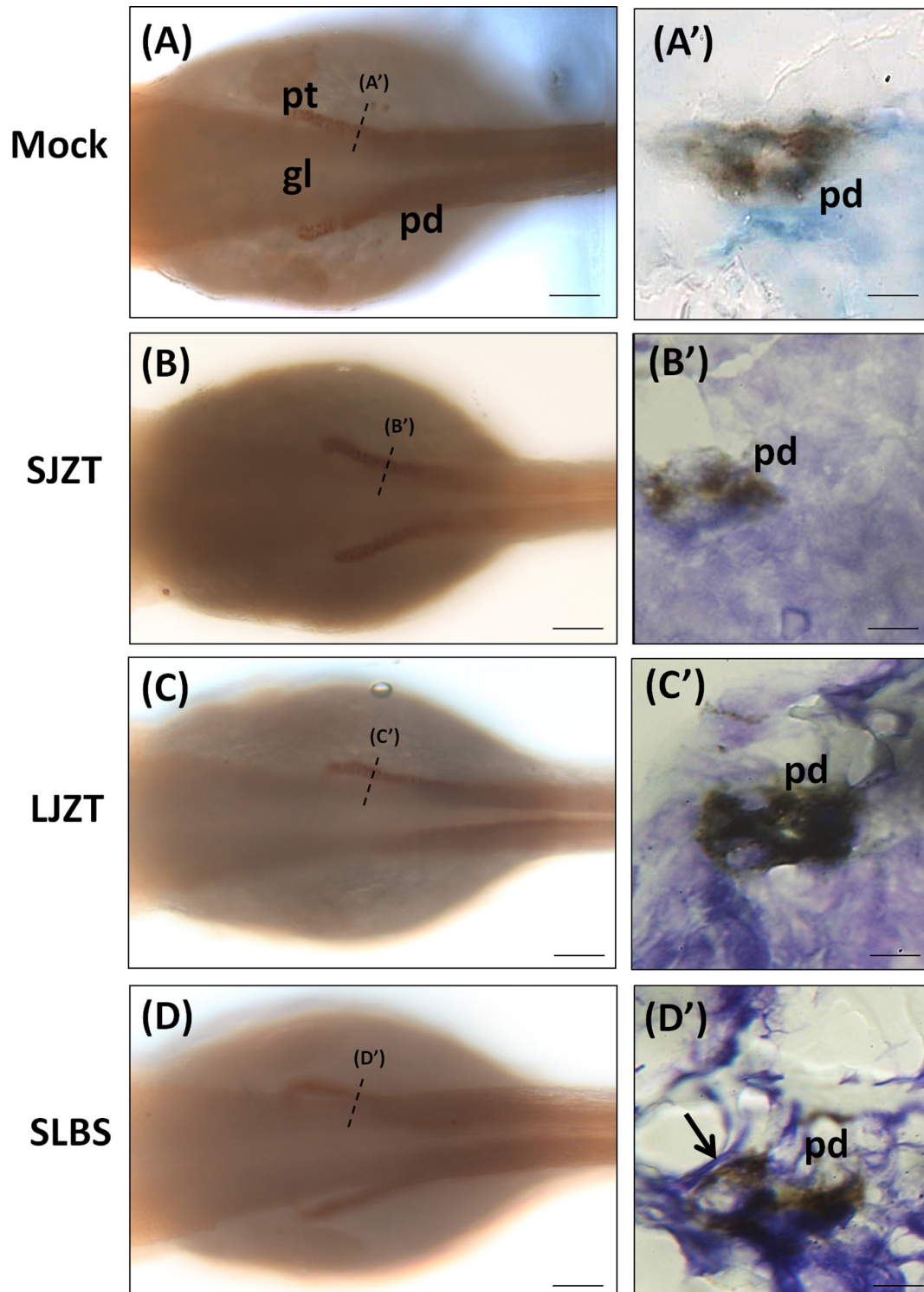
In this study, we demonstrated that the developing ze-

Fig. 4. Immunohistochemical staining of the basolateral marker Na$^+$/K$^+$-ATPase alpha1 subunit (α6F) was performed on transverse sections of the gl, pt and pd from a 48 hpf zebrafish larvae without (A) or with SJZT (B), LJZT (C) and SLBS (D) treatment (250 ng/mL). (A'–D') Sections were stained with a monoclonal antibody against α6F and counterstained with hematoxylin. gl, glomerulus, pd, pronephric duct; pt, pronephric tube. An arrow indicate the defective sites in pd. Scale bar: 40 μm (A–D); 2 μm (A'–D').

brafish kidney was sensitive to CMP (SJZT, LJZT, SLBS) treatment. SLBS was the most toxic (at low dose) in comparison with SJZT and LJZT (Fig. 3). In this regard, it is worthwhile to discuss the reason for the toxicity differences among SJZT, LJZT and SLBS. Our hypotheses are that (1) some common components of SJZT, LJZT and SLBS have nephrotoxic properties and/or (2) some unique component of SJZT, LJZT and/or SLBS might have a different

nephroprotective efficiencies. In the case of nephroprotective effects, after carefully comparing the ingredients of SJZT, LJZT and SLBS, we found that *Panax ginseng* Meyer (ginseng) and *Glycyrrhiza glabra* L. (licorice) are common components of these three CMPs and that *Zingiber officinale* Roscoe (ginger) was present only in SJZT and LJZT, while *Platycodon grandiflorum* (Platycodon root) and *Nelumbo nucifera* Gaertn (lotus) was present only in SLBS. Clinical studies have reported adverse effects (especially organogenesis defects) with overdoses of ginseng and licorice[32–34], whereas both ginger and Platycodon root are able to protect the kidney against cisplatin-induced acute renal failure[35, 36]; *Nelumbo nucifera* Gaertn (lotus) has been reported to have protective effects on carbon tetrachloride-induced injury in mice[37]. Thus, we suggest that the different toxicities of SJZT, LJZT and SLBS might depend on the different pharmacological properties of ginseng, licorice, ginger, Platycodon root and lotus is not only zebrafish but also other species.

In conclusion, CMP-induced kidney defects were easily and dynamically observed *in vivo* during early embryogenesis using the present model. That is, the zebrafish embryo model was demonstrated to be a useful tool to detect the nephrotoxicity of a test substance for mammals. These results could provide novel insights into the subtle changes induced by CMPs that are worthy of further evaluation in higher vertebrates.

Disclosure of Potential Conflicts of Interest: The authors declare no conflict of interest.

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