Evaluation of Tanshinone IIA Developmental Toxicity in Zebrafish Embryos

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Abstract: Tanshinone IIA (Tan-IIA) is derived from the dried roots of Salvia miltiorrhiza Bunge, a traditional Chinese medicine. Although Salvia miltiorrhiza has been applied for many years, the toxicity of the mono-constituent of Salvia miltiorrhiza, tanshinone IIA, is still little studied. This study evaluated the cardiotoxicity and developmental malformations of Tan-IIA by using zebrafish normal embryos and dechorionated embryos. After treatment with Tan-IIA in different concentration for 4 days periods, it is observed obvious pericardial edema, spinal curvature, and even tail missing in zebrafish embryos. The LC_{50} values in dechorionated embryo group at 72 hours post-fertilization (hpf) and 96 hpf were 18.5 μM and 12.8 μM, respectively, and the teratogenicity was manifested at the concentration about 1 μM. The main endpoints of teratogenicity were scoliosis, malformation of tail, and pericardium edema. Our finding displayed the potential cardiotoxicity and severe impact on the abnormal development of Tan-IIA in zebrafish embryo at high concentration, which would help avoid the risk of its clinical application.

Keywords: zebrafish; tanshinone IIA; developmental toxicity

1. Introduction

Tanshinone IIA (Tan-IIA), a fat-soluble fuchsia needle crystal, is the main active ingredients of diterpene quinone in traditional Chinese medicine salvia miltiorrhiza. Because of its unique structure of quinoid, Tan-IIA may involve in multiple biochemical reactions of the organism and have a variety of biological activities. Therefore, Tan-IIA is widely used in the treatment of cardiovascular disease[1-3]. A large number of researches show that Tan-IIA exhibits various pharmacological activities, including anti-inflammatory[4], anti-oxidative[5], anti-fibrosis[6], modulation of collagen metabolism[7], anti-tumor[8] and so on.

Zebrafish (Danio rerio) is a small tropical fish with the advantages of large spawning, rapid breeding, easy to raise, etc[9]. It has been recommended for some standard toxicology test as animal models. Evaluation of drug toxicity using zebrafish has the advantages of easy observation, low cost, simple operation, good repeatability, high sensitivity, and short

experiment period, which is internationally recognized standard method of evaluation of drug toxicity[10]. Zebrafish embryo developmental toxicity is based on teratogenesis and mortality of zebrafish embryo[11, 12]. The current acute toxicity experiments are mainly concentrated in mice as a representative of the animal model. However, due to its high breeding conditions, high cost, complex operation, ethical limits, it is difficult to achieve comprehensive urgent toxicity test[13, 14]. Therefore, using zebrafish model to evaluate drug toxicity is of great significance and zebrafish model has been widely used in the assessment of acute toxicity and developmental toxicity[15-20].

In the previous study, our group explored the acute toxicity of dimethyl sulfoxide (DMSO) by using zebrafish embryo[21], which could be utilized for choosing the safe concentration range of DMSO as a solvent[22]. And this study firstly investigated the developmental toxicity effect of Tan-IIA on the zebrafish embryo. The zebrafish acute toxicity assay is very valid and reliable for rapid evaluation of Tan-IIA toxicity and saves time and cost during drug research and development.

2. Results

2.1. The crystal structure of Tan-IIA

Single crystal X-ray diffraction analysis revealed that Tan-IIA crystallized in the Pmna space group, and the data was summarized Table S1. The fundamental asymmetric group contained only one Tan-IIA molecule. As shown in Figure 1a, two dibenzopyrrole units displayed a large aromatic planar structure, hexamethylene group shown a classic chair conformation. Stacking interactions in a step showed strong overlap for Tan-IIA. The step shown two benzene ring with diketone group occurred with distances to ring centroid of 3.396 Å by π-π stacking interactions. This created an infinite 1D ribbon composed of Tan-IIA secondary building units (SBUs), running along the b axis, as seen in Figure 1d. The layers consisted of benzene ring with diketone group positioned adjacent to each other, separated by 3.396 Å via their π-π stacking interactions, as seen in Figure 1d, e.

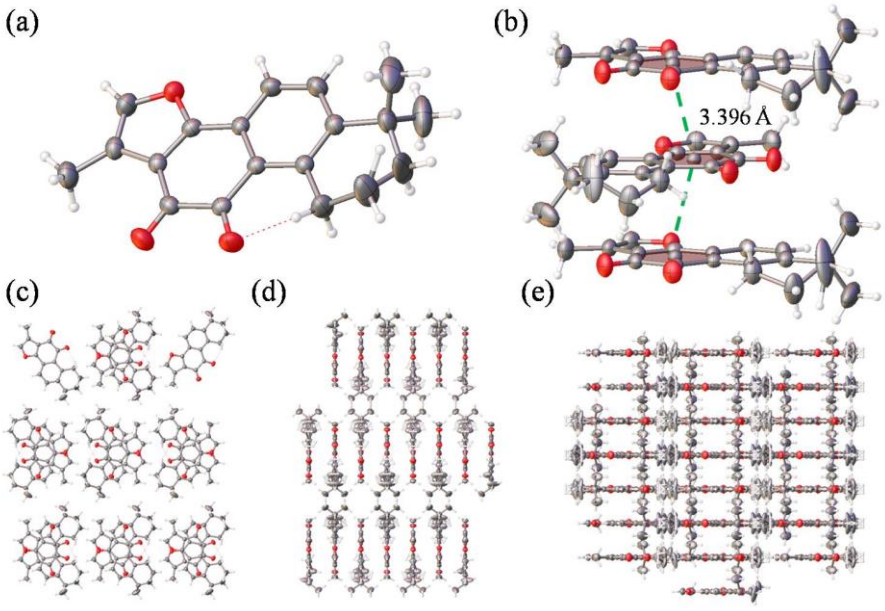

Figure 1. (a) The molecular structure of Tan-IIA. (b) Stacking interactions in a step showing strong overlap for Tan-IIA. The step shown two benzene ring with diketone group stacking interactions occurred with distances to ring centroid of 3.396 Å. The pores viewed along the a axis (c), b axis (d) and c axis (e).

2.2. The lethal effects of Tan-IIA on embryos

The lethal effects were recorded at 24 hpf, 48 hpf, 72 hpf and 96 hpf, as shown in Figure 2. In chorionic embryo group, compared with control group, little obvious lethal effect at the concentration of 1 μM and 3 μM was observed, but with the increasing concentration of Tan-IIA treatment, severe abnormalities of heart and pericardium were occurred, which were observed in a dose-dependent manner. The mortality at 24 μM for 96 hpf just reaching 51.6%.(Figure 2a). While in dechorionated embryo group, lethal effect appeared at the concentration of 5 μM, even reaching 100% mortality (50 μM at 72 hpf and 20 μM at 96 hpf). The LC_{50} values in dechorionated embryo group at 72 hpf and 96 hpf were 18.5 μM and 12.8 μM, respectively (Figure 2b). The hatchability of chorionic embryo group was shown in Figure 2c, which indicated high concentrations of Tan-IIA (12 μM, 24 μM) affected the hatch of embryos. In dechorionated embryo group, the hatchability could not be calculated due to the chorion being removed. The main lethal endpoints observed in dechorionated embryo group were coagulated embryos at 48 hpf and lack of heartbeat at 96 hpf, and lack of somite formation and non-detachment of the tail were also observed in some cases. Above results indicated that Tan-IIA exhibited some toxicity to zebrafish embryo in a dose-dependent manner.

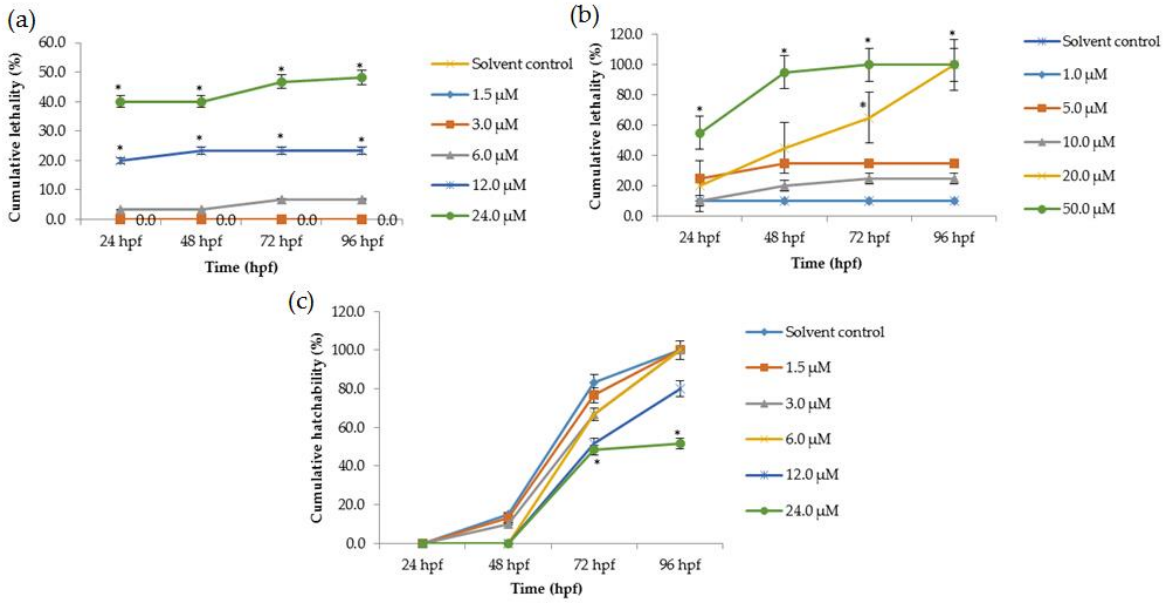

Figure 2. Cumulative lethality curves of embryos exposed to different concentrations of Tan-IIA. (a) Cumulative lethality curves of chorionic embryos; (b) Cumulative lethality curves of dechorionated embryos; (c) Cumulative hatchability curves of chorionic embryos. (n = 20 zebrafish per treatment; *, $P < 0.05$, compared to control)

2.3. The Teratogenic effects of Tan-IIA on embryos

The morphological changes of zebrafish embryos induced by Tan-IIA were further evaluated. Zebrafish have rapidly developed to be a promising model for whole-organism toxicology screening. The influence of Tan-IIA on the development of Zebrafish embryos was determined, as shown in Figure 3, and the detailed data are listed in Table S2.

As shown in Figure 3a, in chorionic embryo group, the zebrafish embryos treating without Tan-IIA had developed normally, but treating with an increasing dosage of Tan-IIA,

the hatched fishes occurred obvious pericardial edema at 6 μM for 96 hpf and spinal curvature at 24 μM for 96 hpf. Moreover, after treating with Tan-IIA ≥ 6 μM, the embryos growing up to fish cost more time than control group, which suggested that Tan-IIA exhibited certain growth inhibition of zebrafish embryos. Besides, in dechorionated embryo group, the zebrafish embryo without chorion could uptake more drugs. It was found that embryos all died at 20 μM for 96 hpf and 50 μM for 72 hpf, and the growth of embryo were inhibited seriously at 50 μM. In a word, above results suggested that Tan-IIA, in a concentration-dependent and low time-dependent manner, exhibited certain toxicity and growth inhibition to zebrafish embryos *in vivo* at high concentration.

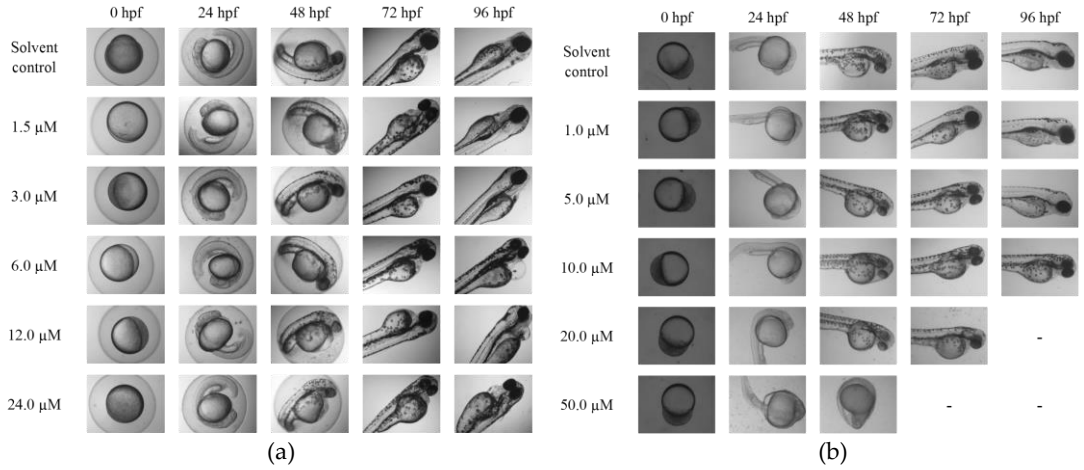

Figure 3. Morphology of zebrafish embryos exposed to Tan-IIA. (a) Morphology of chorionic embryos; (b) Morphology of dechorionated embryos. -: all dead.

Moreover, with the protection of chorion, the fishes hatched from eggs exposed to different concentrations (0, 1.5 and 3 μM) of Tan-IIA for 96 hpf displayed great health conditions, but the zebrafish treated with 6 μM showed abnormal development with pericardial edema and scoliosis, even tail missing at 24 μM. However, without protection of chorion, the fishes occurred mass mortality and mild teratogenic effects at 5 μM for 96 hpf. The all major malformations of scoliosis, tail autolysis, and pericardial edema were observed at the highest concentration of 50 μM (Figure 4b). Especially in groups with 20 μM and 50 μM of Tan-IIA, the zebrafish pericardium was enlarged, edematous and congested, which appeared about two-times larger than that of control group. These results indicated that Tan-IIA exhibited potential cardiotoxicity and growth inhibition, which implied that Tan-IIA was unsuitable for pregnant women, baby and kids.

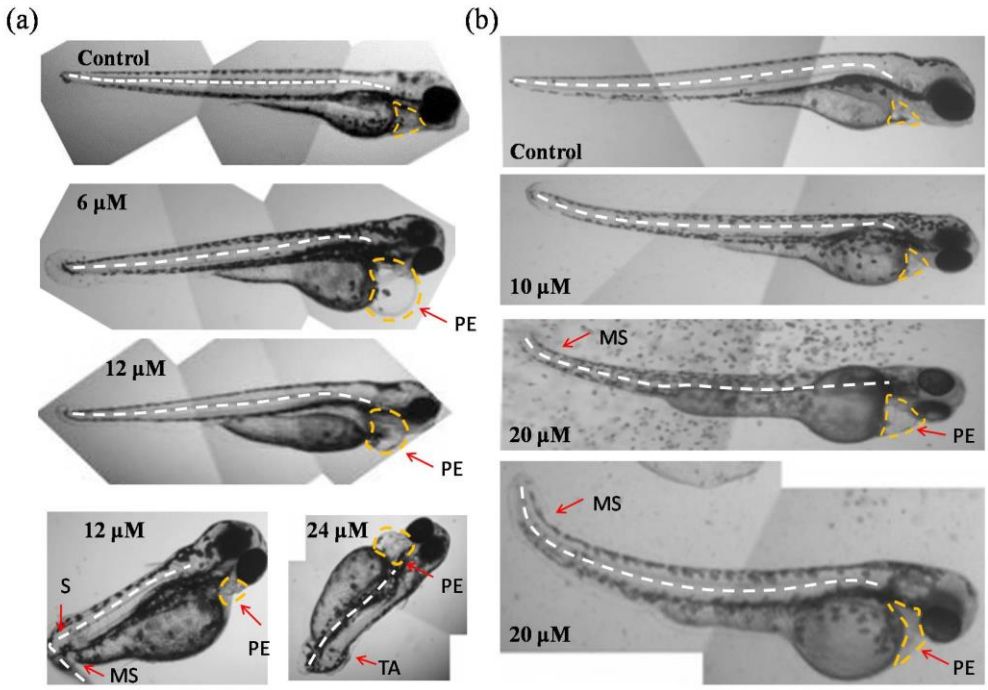

Figure 4. Abnormal embryos exposed to Tan-IIA. (a) Abnormal embryos in chorionic embryo group; (b) Abnormal embryos in dechorionated embryo group. S: scoliosis; PE: pericardial edema; TA: tail autolysis; MS: malformation of spine.

3. Discussion

Although *Salvia miltiorrhiza* has been applied for many years, the toxicity of the mono-constituent of *Salvia miltiorrhiza*, tanshinone IIA, is not been fully studied. Cao Xiaomei et al.[23] used cavy and rabbit to evaluate the hemolytic effect, hormesis effect and anaphylaxis of sodium tanshinone IIA sulfonate injection. Our research explored the developmental toxicity and acute toxicity of Tan-IIA by using chorionic embryos and dechorionated embryos. The lethality and teratogenicity were observed and summarized at different concentrations of Tan-IIA.

The chorion of zebrafish embryo is the acellular envelope surrounding embryo. The role of the chorion is to protect embryo from damage and to prevent polyspermy[24, 25]. As shown in Figure 2a, 2b, the lethality of the chorionic embryo group was lower than dechorionated embryo group at the similar concentration of Tan-IIA. In dechorionated embryo group, the LC_{50} values at 72 hpf and 96 hpf were 18.5 μM and 12.8 μM, respectively, while in chorionic embryo group, the lethality of the highest concentration (24 μM) was below 50%. The results indicated that Tan-IIA may be partly blocked by the chorion. The incubation of zebrafish embryo is the result of hatching enzymes and mechanical force[26]. In the process of hatch, the chorion is decomposed by hatching enzymes, and then the embryo with warp and sway would break through the chorion[27, 28]. As shown in Figure 2b, the hatchability was declined at the concentration of 12 μM and 24 μM, which revealed that Tan-IIA of high concentration might affect the functions of hatching enzymes and/or motor system of embryo.

Development of zebrafish is very similar to mammals in most aspects of embryo development, including early embryonic processes, and development of cardiovascular, somite and skeletal, etc[11, 12]. When the embryos are exposed to chemicals upon fertilization, teratogenic effects could be observed in the following several days. As shown in Figure 4 and Table S3, there were no teratogenic effects as the concentration of Tan-IIA was below 5 μM in both chorionic and dechorionated embryo groups. The major malformations observed were

scoliosis, tail autolysis, and pericardial edema. Due to the resistance function of chorion, the appearance of teratogenic effect was delayed compared to dechorionated embryo group.

4. Materials and Methods

4.1. Materials and Reagents

Tanshinone IIA (Tan-IIA) was purchased from Damao Chemical Reagent Factory (Tianjin, China), protease E from Sigma-Aldrich (Guangzhou, China), dimethyl sulfoxide (DMSO) from Guangzhou Chemical Reagent Factory (Guangzhou, China), 6-well plate from Guangzhou JET Bio-Filtration Co., Ltd (Guangzhou, China), inverted optical microscope from Chongqing Optec Instrument Co., Ltd (Chongqing, China).

4.2. Brood Zebrafish Maintenance and Egg Production

Zebrafish were maintained according to the book of *The Zebrafish Book*[29]. Wild type Tuebingen strain zebrafish (Danio rerio) were obtained from China Zebrafish Resource Center, CZRC (Wuhan, China), and 3-months-old zebrafish was used for egg production. Sexually mature zebrafish were maintained in a recirculating aquaculture system (Shanghai Haisheng Biotech Co., Ltd, China) at 28.0 ± 1.0 °C. The water was purified by water purifier (Shenzhen Luoke Water Purification Equipment Co., LTD, China), and the $NaHCO_3$ and NaCl were used to adjust the pH and conductivity at pH 6.8~7.4 and 500~550 μS, respectively. The housing system is equipped with temperature control unit, UV light and activated carbon filter system. The day to night photoperiod was 14 h: 10 h. Mature zebrafish were fed with brine shrimp twice and powder feed once daily.

Males and females at a ratio of 2: 1 or 1: 1 were placed in spawning tanks a few hours before the onset of darkness on the day prior to the test. For the collection of eggs, spawn traps were placed into the spawning tanks, and the spawn traps were covered with inert wire mesh of appropriate mesh size. About 30 min after the onset of light, spawn traps were removed and eggs were collected. To avoid genetic bias, eggs are collected from a minimum of three breeding groups, mixed and randomly selected.

4.3. The Developmental Toxicity Assay

The developmental toxicity assay was carried out according to the fish embryo acute toxicity (FET) test of OECD guidance[30] and the book of *Zebrafish: Methods for Assessing Drug Safety and Toxicity* with modification[31, 32].

4.3.1. Removal of Chorion

The method of removing chorion referred to the published protocol[33]. Eggs were placed in 90-mm petri dish and the water was sopped up. 5 ml of pronase (0.208 g/L, warmed to 28.5 °C) was poured into the petri dish and incubated for 6 min. 20 ml fish water was add into the petri dish, and let the eggs sink to the bottom of the petri dish, then slowly pouring the water out. The eggs were gently rinsed three times with fish water and most of the chorions had been removed.

4.3.2. Embryo Exposure

In the preliminary experiment, the concentrations of Tan-IIA of 1.5 μM, 3.0 μM, 6.0 μM, 12.0 μM, 24.0 μM were selected. Tan-IIA was dissolved in DMSO with the final solvent concentration of 0.2% in the test solution. 5 ml of these solutions and 20 chorionic embryos of 2 h post-fertilization (hpf) were transferred to 6-well plates. Each test concentrations and solvent control were on the same plate, setting three parallel experimental plates and one negative

control plate of fish water. The embryos were incubated at 28.0 ± 1.0 °C with the photoperiod of 14 h: 10 h.

According to the results of the pre-test, the concentrations of Tan-IIA were changed as 1.0 μM, 5.0 μM, 10.0 μM, 20.0 μM, and 50.0 μM with the final DMSO concentration of 0.4% in the test solution. In addition, normal dechorionated embryos of 2 h post-fertilization (hpf) were used for exposure. The other methods were the same as preliminary experiment above.

4.3.3. Observation of Mortal and Teratogenic Effects

Observations were recorded using inverted optical microscope every 24 h, until the end of the test, including coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. Any positive outcomes in one of these observations mean that the zebrafish embryo was dead. The teratogenic embryos were photographed to analyze the deformed parts. LC_{50} were calculated at 72 hpf and 96 hpf.

4.4. Statistical Analysis

The experimental data are expressed as the mean ± SD (standard deviation). The significance of mortality and teratogenesis rates were determined by chi-square (χ^2) test and Fisher's exact test. Significant differences were considered at $P < 0.05$. Statistical analysis was conducted using SPSS 17.0.

5. Conclusions

Tanshinone IIA, the main active ingredients of *salvia miltiorrhiza*, which is an important drug to treat disease of cardiovascular system, such as hypertension and atherosclerosis. The in vitro potential toxicity of Tan-IIA was evaluated by zebrafish embryo model. It was found that Tan-IIA exhibited severe growth inhibition, development malformation and cardiotoxicity at high concentration, which stated that Tan-IIA was inappositely taking by the growing crowd of pregnant women, baby and kids. This study firstly reported the potential toxicity of Tan-IIA, pointing out more potential risk of its clinical application and providing the help for the instruction of clinical medication.

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Author Contributions: Tao Wang and Chengxi Wang conceived and designed the experiments; Tao Wang and Jiaojiao Chen performed the experiments; Tao Wang and Hao Zhang analyzed the data; Yutao Lan and Xicheng Wang contributed materials and analysis tools; Tao Wang wrote the paper; Baoguo Wang and Wenjie Mei revised the paper. All the authors read and approved the final manuscript.

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

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Sample Availability: Samples of the compounds are available from the authors.