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Research article

Exposure to tris (1,3-dichloro-2-propyl) phosphate affects the embryonic cardiac development of *Oryzias melastigma*

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ABSTRACT

Tris (1,3-dichloro-2-propyl) phosphate (TDCPP) is a growing concern and may be a potential risk to marine environmental health due to its widespread usage and distribution. However, the toxic effects of TDCPP on cardiac development in marine fish have not been reported. In this study, *Oryzias melastigma* embryos were exposed to TDCPP at doses of 0, 0.04, 0.4, 4 and 40 μg/L from early embryogenesis to 10 days postfertilization (dpf). Then, the heart rate and sinus venosus–bulbus arteriosus (SV–BA) distance of the exposed embryos were measured at 5, 6, 8 and 10 dpf. Furthermore, alterations in the mRNA levels of the genes encoding cyclooxygenase-2 (*COX-2*), bone morphogenetic protein 4 (*BMP4*), fibroblast growth factor 8 (*FGF8*), and GATA-binding protein 4 (*GATA4*) were evaluated at 5, 6, 8 and 10 dpf. We found that the heart rate significantly increased in all TDCPP exposure groups at 10 dpf. The SV–BA distance significantly decreased in all TDCPP exposure groups at all developmental stages (except for the 0.4 μg/L group at 5 dpf and the 4 μg/L group at 10 dpf). The mRNA expression of *COX-2* was downregulated at 5 dpf, *BMP4* was downregulated at 5 and 6 dpf, *FGF8* was downregulated at 5, 6 and 8 dpf, *GATA4* was downregulated at 8 dpf, and *GATA4* was upregulated at 10 dpf. These results indicate that the changes in heart rate and SV–BA distance might be accompanied by disturbances in the four genes involved in cardiac development. Our findings will help to illustrate the possible cardiac toxic effects of marine fish exposed to TDCPP.

1. Introduction

Polybrominated diphenyl ethers (PBDEs), applied to prevent fire or diminish the chance of ignition, have been widely used commercially and industrially in recent decades [1,2]. Due to their proven toxicity, bioaccumulation and persistence, PBDEs are being phased out of production [3,4]. As one of the major replacements for PBDEs, tris (1,3-dichloro-2-propyl) phosphate (TDCPP) has been frequently used as a flame retardant in electronics, foams, plastics, textiles, paints and furniture [4,5]. However, recent studies have

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shown that TDCPP can easily leach out into the environment and thus is considered the highest priority for aquatic life risk assessment [5–9]. Environmental monitoring has shown that TDCPP is frequently detected in air, indoor dust, surface water [10], drinking water [11], sediment [12], and wildlife and human tissues [4,13]. TDCPP at different concentrations has been detected in water environmental media worldwide. For example, the concentration of TDCPP in freshwater reached 1.34 μg/L in Albano Lake, Italy [14], and that in seawater was 0.032–0.38 μg/L in Lianyungang, China [15]. In the sewage of a laundromat in the USA, the TDCPP concentration reached 65.6 μg/L [16].

Previous studies have found that TDCPP has various toxic effects in freshwater fish, including inhibition of growth and development [17–20], reproductive toxicity [4], neurotoxicity [21,22], and hepatotoxicity [23]. For example, in *Danio rerio*, TDCPP exposure increased malformation and altered the normal trajectory of early embryogenesis [17,20,24]. TDCPP had widespread effects on neurotrophic factor genes in *Gobiocypris rarus* [25]. Exposure to TDCPP was found to significantly reduce the body length of female *D. rerio* [26]. TDCPP exposure induced mRNA expression of genes related to phase I and II metabolic enzymes in *D. rerio* liver [27].

The cardiovascular system has been shown to be the first functioning component of developing vertebrate embryos [28,29]. Numerous previous studies have shown that fish hearts are highly sensitive to pollutants. For example, heart dysfunction was observed in embryos of *D. rerio* exposed to phenanthrene (Phe) [30]. TDCPP could cause severe pericardial edema in the early stages of *D. rerio* embryonic development [17]. Huang and his colleagues found that bisphenol A (BPA) could change the sinus venosus–bulbus arteriosus (SV–BA) distance and the expression of cardiac development-related genes in *Oryzias melastigma* [31]. Environmentally relevant concentrations of hexabromocyclododecane diastereoisomers increased the heart rate of *O. melastigma* embryos [32]. However, to our knowledge, information about the cardiac toxicity caused by TDCPP exposure in marine fish and the underlying mechanism is lacking. Thus, a marine fish model is urgently needed to further study the specific effects of TDCPP.

O. melastigma is an excellent candidate in toxicological studies, because of its small size, short generational time, large number of offspring per laying, and transparency of eggs and embryos. Therefore, *O. melastigma* has been widely used in marine ecological toxicology studies [28,33–35]. For *O. melastigma*, the embryo develops a regular heartbeat at 5 days postfertilization (dpf), shows the highest heart rate at 8 dpf, and achieves a constant heart rate at 11 dpf [36].

Since there is an urgent need to know whether exposure to TDCPP may lead to cardiac toxicity in marine fish, we employed the *O. melastigma* model to assess the cardiac toxicity of embryos caused by TDCPP exposure in the present study. The heart rate and SV–BA distance were examined to confirm whether TDCPP exposure adversely affected heart development, and the expression of several cardiac development-related genes was determined to further explore the mechanism involved. This work will improve our understanding of the potential toxicity of TDCPP in marine fish.

2. Materials and methods

2.1. Chemicals

TDCPP (CAS: 13674-87-8; >97 % purity) and dimethyl sulfoxide (DMSO; >99.9 % purity) were purchased from Sigma–Aldrich (Shanghai, China). TDCPP was dissolved in DMSO for stock concentrations of 4, 40, 400 and 4000 mg/L.

2.2. Embryo maintenance and toxicity tests

O. melastigma was obtained from the City University of Hong Kong and bred for many generations in our laboratory. *O. melastigma* was maintained with aerated artificial seawater of 30 ‰ salinity and kept at 28 ± 2 °C in a 14:10 h light: dark photoperiod cycle. The fish were fed twice daily with live brine shrimp. Food residues and excrement were removed, and the artificial seawater was renewed every day.

Embryos were collected within 2 h postfertilization and were visually sorted under an XTZ-D stereomicroscope (SunGrant, Shanghai, China). The dead and unfertilized embryos were removed. According to the reported environmentally relevant concentration (e.g., 0.032–0.38 μg/L [15] or 65.6 μg/L [16]), and exposure concentrations (e.g., 10–200 μg/L [37] or 5.66–92.8 μg/L [38]), the exposure concentrations of TDCPP used in our study were 0.04, 0.4, 4 and 40 μg/L. Forty embryos were randomly incubated in 90 mm petri dishes containing 30 mL of seawater with TDCPP. The same number of embryos were used as controls that were not exposed to TDCPP. The final DMSO concentrations in each exposure group and the control group were all 10 μL/L. Five replicates were used for each group, and the exposure solution was renewed every day. The culture conditions were the same as those mentioned above. The Animal Care and Use Committee of the Key Laboratory of Mariculture & Stock Enhancement in North China's Sea at Dalian Ocean University approved all fish-related procedures in this study.

2.3. Quantification of TDCPP in embryos

The TDCPP concentration in the embryos ($n = 3$ Eppendorf tubes, 120 embryos per tube) was measured at all concentrations at 10 dpf, as described previously [37,39]. Specifically, the embryos were homogenized with acetonitrile. Then, the mixed solution was sonicated and centrifuged. The supernatant was collected and sonicated two additional times using acetonitrile. The eluate was concentrated to 1 mL under a steady N_2 flow. Liquid chromatography-tandem mass spectrometry analyses of TDCPP were performed with a high performance liquid chromatography system (LC-20ADXR, Shimadzu, Japan) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer (5500 QTRAP, AB Sciex, USA). TDCPP standards (0.1, 0.5, 1, 2, 5, 10 and 20 ng/mL) were prepared in acetonitrile and used to develop the calibration curve. TDCPP was detected as an ion with a molecular weight of 430.9.

2.4. Assessment of TDCPP-induced cardiac toxicity

The survival rate of embryos ($n = 5$ petri dishes, 40 embryos for each dish) was measured after TDCPP exposure at all concentrations at 10 dpf. The heart rate and SV–BA distance of the embryos ($n = 5$ petri dishes, 5 embryos for each dish) were measured at all concentrations at 5, 6, 8 and 10 dpf. The heart rate was directly determined under a fluorescence microscope (Leica, Wetzlar, Germany) during a 20 s period. The SV–BA distance was measured using the two-dimensional measuring software (Image-Pro Plus, Media Cybernetics, USA), according to the reported method [28,40]. In brief, the distance of SV–BA was presented as the length of a straight line connecting the two structures on acquired images under the same amplification factor and in similar observation sessions.

2.5. Quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted from embryos (n = 4 tubes, 30 embryos per tube) at all concentrations at 5, 6, 8 and 10 dpf using TRIzol reagent (TaKaRa, Beijing, China). The OD ratio (260:280) of RNA was between 1.781 and 2.091 (Supplementary Table S1). The 28S electrophoresis band was approximately twice as bright as the 18S electrophoresis band (Supplementary Fig. S1). One microgram of total RNA was reverse-transcribed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Beijing, China) following the protocols given in the kit. The primers designed by Huang et al. [28] were used to amplify the four cardiac development-related genes, cyclooxygenase-2 (*COX-2*), bone morphogenetic protein 4 (*BMP4*), fibroblast growth factor 8 (*FGF8*) and GATA-binding protein 4 (*GATA4*) (Supplementary Table S2). qRT-PCR analyses were performed on an ABI StepOnePlus™ Real-Time PCR System with Tower and MonAmp™ ChemoHS qPCR Mix (Mona, Wuhan, China). The qRT-PCR conditions were as follows: initial denaturation at 95 °C for 10 min; 40 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 30 s; and finally, analysis of the dissociation curve. The mRNA levels of target genes were normalized to that of *18S* (Supplementary Table S2). The $2^{-\Delta\Delta CT}$ method was further used to calculate the relative expression of target gene mRNA.

2.6. Data analysis

Statistical analysis of the data was performed using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA followed by Duncan's post hoc test. $P < 0.05$ was considered significant. Data are presented as the means ± SE.

3. Results

3.1. Concentration of TDCPP in embryos

The TDCPP concentrations in embryos of the 0.04, 0.4, 4 and 40 μg/L groups at 10 dpf were 694.12 ± 34.93, 920.27 ± 10.01, 1061.65 ± 77.46, and 1860.84 ± 452.06 ng/kg (wet weight; all $n = 3$), respectively. No TDCPP was detected in the control group (Table 1).

3.2. Effects of TDCPP on embryo survival

No significant differences in the survival rate (Fig. 1), hatching rate (Supplementary Fig. S2), hatching time (Supplementary Fig. S3), or malformation rate (Supplementary Fig. S4) of embryos were observed among the different exposure groups compared with the control group (all $P > 0.05$).

3.3. Effects of TDCPP on heart rate

No significant difference in heart rate was observed in any of the exposure groups compared with the control group at 5, 6 and 8 dpf (all $P > 0.05$; Fig. 2). At 10 dpf, a significant increase in the heart rate was observed in all the exposure groups compared with the control group (all $P < 0.05$; Fig. 2).

3.4. Effects of TDCPP on the SV–BA distance

A representative picture depicting the SV–BA distance is shown in Fig. 3A. The SV–BA distance significantly decreased after

Table 1
TDCPP concentration in embryos of the five groups.

Group (μg/L)	TDCPP concentration in embryos at 10 dpf (ng/kg)
0	0
0.04	694.12 ± 34.93
0.4	920.27 ± 10.01
4	1061.65 ± 77.46
40	1860.84 ± 452.06

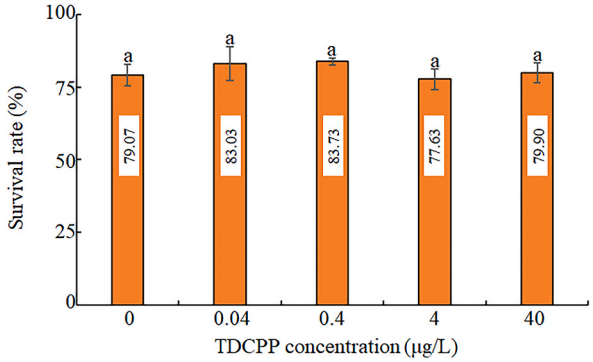

Fig. 1. Cumulative survival of embryos after exposure to different concentrations of TDCPP. The results are presented as the means ± SE ($n = 5$, each replicate included 40 embryos). The different letters indicate significant differences among different treatments ($P < 0.05$).

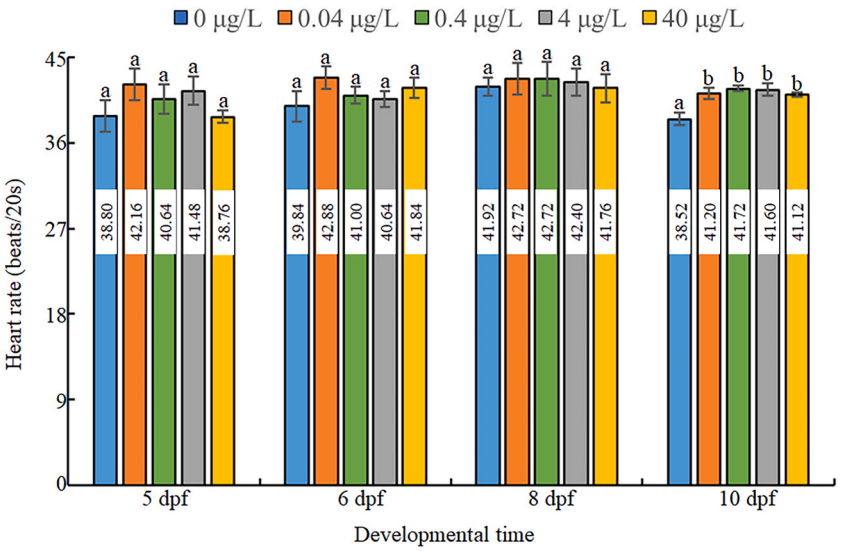

Fig. 2. Heart rate of embryos at different developmental stages after exposure to different concentrations of TDCPP. The results are presented as the means ± SE ($n = 5$, each replicate included 5 embryos). The different letters indicate significant differences among different treatments ($P < 0.05$).

treatment with TDCPP at all exposure concentrations at all developmental stages (except for 0.4 μg/L at 5 dpf and 4 μg/L at 10 dpf) compared with that of the control group (all $P < 0.05$; Fig. 3B).

3.5. *Effects of TDCPP on cardiac development-related gene expression*

The results of cardiac development-related gene expression are shown in Fig. 4 and Supplementary Table S3. The expression of *COX-2* was significantly downregulated in all the exposure groups at 5 dpf ($P < 0.05$; Fig. 4A). There was no significant difference among the different exposure groups at 6, 8 and 10 dpf (all $P > 0.05$; Fig. 4A). The expression of *BMP4* was significantly downregulated in the 4 μg/L group at 5 dpf and in the 0.4 μg/L group at 6 dpf (all $P < 0.05$), but a significant difference was not observed among the different groups at 8 and 10 dpf (all $P > 0.05$) compared with the control group (Fig. 4B). The expression of *FGF8* was significantly downregulated in all the exposure groups at 5 dpf, in the 0.4, 4 and 40 μg/L groups at 6 dpf and 8 dpf (all $P < 0.05$), but a significant difference was not observed in any group ($P > 0.05$) compared with the control group at 10 dpf (Fig. 4C). The expression of *GATA4* was significantly downregulated in the 0.4, 4 and 40 μg/L groups at 8 dpf ($P < 0.05$), but a significant difference was not observed among the different groups (all $P > 0.05$) compared with the control group at 5 and 6 dpf (Fig. 4D). Furthermore, a significant increase was observed in all the exposure groups compared with the control group at 10 dpf ($P < 0.05$; Fig. 4D).

4. Discussion

Cardiac development, an extremely elaborate and sensitive process, is susceptible to anomalies because of the precisely coordinated cellular proliferation, migration, differentiation and complex morphogenetic interactions involved [41,42]. Moreover, cardiac

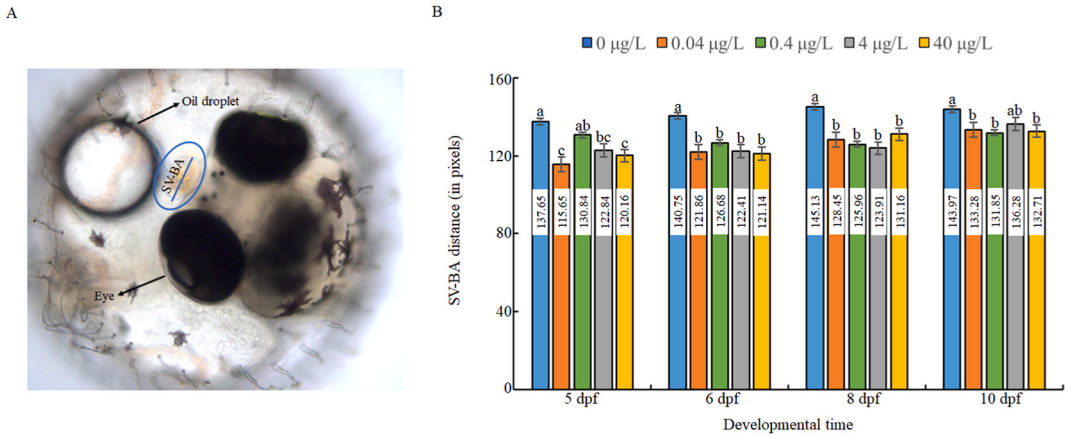

Fig. 3. SV–BA distance of embryos at different developmental stages after exposure to different concentrations of TDCPP. A representative micrograph of the embryo (A). The heart region is circled, and the SV–BA distance is marked by a bule line. Statistical differences in the SV–BA distance (B). The results are presented as the means ± SE ($n = 5$, each replicate included 5 embryos). The different letters indicate significant differences among different treatments ($P < 0.05$).

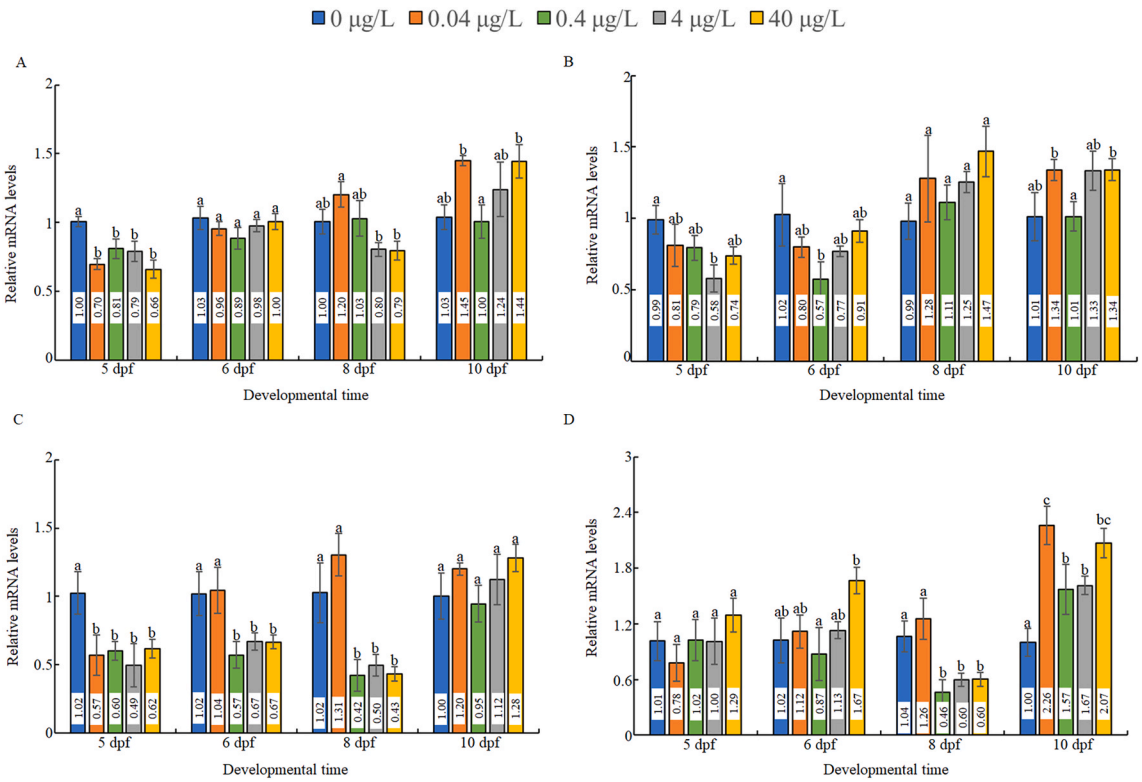

Fig. 4. The expression of four cardiac development-related genes in embryos at different developmental stages after exposure to different concentrations of TDCPP. *COX-2* (A); *BMP4* (B); *FGF8* (C); *GATA4* (D). The results are presented as the means ± SE, ($n = 4$, each replicate included 30 embryos). The different letters indicate significant differences among different treatments ($P < 0.05$).

development can be affected by various toxic chemicals (e.g., persistent organic pollutants, heavy metals, pesticides, fungicides) during embryonic development in fish [18,41,43,44]. For instance, *D. rerio* embryos exposed to Phe had abnormally looped and dilated hearts [30]. Embryonic exposure to crude oil could cause circulatory disruption, pericardial edema and other secondary malformations of the heart in tunas and swordfish [45]. TDCPP can cause a variety of toxicities in fish, such as developmental toxicity, neurotoxicity, and hepatotoxicity [46,47]. However, to the best of our knowledge, research on the toxicity of TDCPP to fish has mainly focused on freshwater fish [4,20,25,26,46]. Few marine fish have been studied to assess the cardiac toxicity of TDCPP, which has been detected in

seawater [15,48]. In this study, we used the *O. melastigma* embryo model to assess the toxicity of TDCPP on cardiac development for the first time.

Previous studies have found that fish exposed to TDCPP could bioaccumulate TDCPP [37,39]. In our study, the TDCPP contents in embryos exposed to this chemical increased with the exposure concentration, indicating that TDCPP exposure caused bioaccumulation in *O. melastigma* embryos. In addition, the possibility for contaminant uptake and transport to cardiovascular tissues in the early life stage of fish is high [49]. We speculated that TDCPP might have direct effects on heart development by entering the heart of *O. melastigma* embryos.

The survival rate of *O. melastigma* embryos was not significantly different among the different TDCPP treatment groups, which is consistent with a previous report that found that the survival rate was not significantly affected by TDCPP exposure at concentrations up to 200 μg/L [37]. Although exposure to TDCPP did not affect the survival rate of *O. melastigma* embryos in our study, heart development was significantly affected.

A previous study showed that the most affected physiological parameter of cardiac function in *O. melastigma* embryos is the heart rate [50]. The heart rate is a powerful predictor of death from either cardiovascular or noncardiovascular causes [51]. A high heart rate could increase the risk of sudden cardiac death and heart failure [52]. In our study, the heart rate of *O. melastigma* embryos was significantly increased only at 10 dpf in all exposure groups and alterations occurred in the late developmental stage. A recent study also found that heart rate increased in the late stage of embryonic development after exposure to the herbicide prometryn in *O. melastigma* [44]. In future studies, researchers need to clarify whether this increased heart rate of *O. melastigma* embryos caused by TDCPP exposure could further affect the survival of larvae and adults. Another report showed that the increased distance of SV–BA could decrease the heart rate in *D. rerio* embryos after 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure [40]. In our study, we hypothesized that the decreased SV–BA distance might be one of the reasons for the increased heart rate in *O. melastigma* embryos. In addition, previous studies have confirmed that changes in calcium homeostasis in cardiomyocytes could lead to arrhythmia [53,54]. We hypothesized that calcium homeostasis might play a role in regulating the cardiotoxicity caused by TDCPP in *O. melastigma* embryos. To confirm these hypotheses, researchers must study the molecular mechanism of the increased heart rate caused by TDCPP in the future.

The SV is the junction where blood flows into the atrium, while the BA is the junction where blood flows out from the ventricle. The SV–BA distance reflects the change in cardiac looping and has been used as a crucial index for the evaluation of cardiac development [40]. Previous studies have reported that poisons could alter the distance of SV–BA and affect heart development in *O. melastigma* [31,32]. In our study, the SV–BA distance was decreased by TDCPP at all concentrations in all developmental stages (except for 0.4 μg/L at 5 dpf and 4 μg/L at 10 dpf), showing that the SV–BA distance was sensitive to TDCPP exposure.

To reveal the molecular changes in heart development after TDCPP exposure, we determined the expression of four cardiac development-related genes, *COX-2*, *BMP4*, *FGF8* and *GATA4*. *COX-2* is related to the inflammatory response in the cardiovascular system [55], and is mainly expressed in the heart [56]. The high expression of *COX-2* could increase the SV–BA distance in *O. melastigma* embryos [31]. In our study, *COX*-2 mRNA expression was downregulated at 5 dpf in all concentration groups. Thus, we speculate that the decreased SV–BA distance might be related to the decreased expression of *COX-2*. *BMP4* is a crucial regulator of early heart development and is used to assess cardiac toxicity [44,57,58]. *BMP4* expression was observed in the heart, brain, digestive tracts, testes, and jaw [59]. *BMP4* mRNA expression was downregulated at 5 dpf in the 4 μg/L group and 6 dpf in the 0.4 μg/L group, indicating that TDCPP affects the heart development of *O. melastigma* embryos. A similar study showed that exposure to perfluorooctane sulfonate reduced the expression of *BMP4* in *O. melastigma* embryos [28]. *FGF8* was also found to be expressed in the heart [56]. This gene is essential for heart precursor development [60]. Downregulation of *FGF8* caused cardiac dysmorphogenesis in *O. melastigma* exposed to prometryn [44]. Our results showed that the mRNA expression of *FGF8* was downregulated at 5 dpf in all concentration groups; 6 dpf in the 0.4, 4 and 40 μg/L groups; and 8 dpf in the 0.4, 4 and 40 μg/L groups. We presumed that the downregulation of *FGF8* might be correlated with the malformation of *O. melastigma* heart development after TDCPP exposure. *GATA4* is a zinc finger transcription factor that regulates genes involved in embryogenesis and myocardial differentiation and function [61]. Lai et al. [62] found that *GATA4* was expressed in the heart and ovaries of *O. melastigma*. We found that the mRNA expression of *GATA4* was downregulated at 8 dpf in the 0.4, 4 and 40 μg/L groups. A similar study showed that exposure to BPA reduced the expression of *GATA4* in *O. melastigma* embryos [31]. Generally, the expression of *GATA4* contributes to regeneration in damaged cells in response to injury in the developing heart [63]. The decreased expression of *GATA4* indicated that the repair of heart injury in *O. melastigma* embryos could be affected by TDCPP exposure, which might be one of the reasons for the cardiotoxicity caused by TDCPP. Notably, the mRNA expression of *GATA4* was upregulated at 10 dpf in all concentration groups. The increased expression of *GATA4* indicated that the embryo likely repairs heart injury after TDCPP exposure for 10 days. We found significant changes in the expression of these four genes in the 0.4 and 4 μg/L TDCPP groups, indicating that the expression of *COX-2*, *BMP4*, *FGF8* and *GATA4* was more easily affected at 0.4 and 4 μg/L TDCPP. These findings suggest that the expression changes in these genes might be internal causes of cardiotoxicity induced by TDCPP. Due to the complexity of the transcriptional regulatory network in heart development, changes in the expression of limited regulators might not fully explain the mechanism of cardiotoxicity. More members of this network and their spatiotemporal expression should be investigated in future studies. Notably, the exact relationship between the expression of cardiac development-related genes and functional loss of the heart was not firmly established in this study, thus more researches are needed to explore these relationships in the future. As the embryo is very small in the early developmental stages of *O. melastigma*, it is difficult to dissect the heart to explore cardiotoxicity. Additionally, many previous cardiotoxic studies used whole embryos to investigate the expression of cardiac development-related genes in fish [44,56,64]. Therefore, the whole embryo was used to examine the expression of these genes in our study.

Dose-dependent effects on heart rate, SV–BA distance, and gene expression were not observed in our study. One possible reason is that the exposure time was not long enough [65]. To confirm this conclusion, researchers should perform a long-term exposure

experiment. Embryonic period is a very sensitive window, TDCPP exposure might cause defect in other organs of *O. melastigma* embryos. More researches are needed to study other toxicities on *O. melastigma* embryos in the future.

5. Conclusion

In conclusion, our study is the first to reveal the cardiac developmental toxicity of TDCPP on *O. melastigma*. TDCPP exposure increased the heart rate, resulted in a narrowed SV–BA distance, and modulated the expression of four cardiac development-related genes (*COX-2*, *BMP4*, *FGF8* and *GATA4*). These genes might be involved in the progression of TDCPP-induced cardiac development toxicity, and further studies are needed to elucidate the toxic mechanisms. Considering the development of industrialization and human activities, the TDCPP concentration in the environment might increase. The increasing TDCPP concentration means that the threat to the aquatic environment and marine ecosystem is becoming increasingly serious. Therefore, our study will be helpful in assessing the toxicity of TDCPP in marine fish.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Chenshi Wang: Writing – original draft, Investigation, Data curation, Methodology. **Wei Lei:** Writing – original draft, Data curation, Conceptualization, Validation. **Chengchen Jiang:** Investigation, Methodology. **Lichao Du:** Investigation, Methodology. **Xindi Huang:** Investigation, Data curation. **Xiaoyu Cui:** Investigation, Data curation. **Dongxu Gao:** Writing – review & editing, Project administration, Investigation, Methodology. **Hua Wang:** Writing – review & editing, Conceptualization, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25554.

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