Toxicity Effects of the Environmental Hormone 4-Tert-Octylphenol in Zebrafish (Danio rerio)

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Abstract

4-tert-octylphenol (4-t-OP), an environmental exogenous estrogen is produced by microbial degradation of alkylphenol polyethoxylates (APEOs). Although it is well known that 4-t-OP can cause the feminization of male, sterility and deficiency of gonad development of aquatic animals by disrupting the endocrine reproductive signaling, less is known about the effects of 4-t-OP on embryonic development. Moreover, the presence of 4-t-OP were detected in umbilical cord blood samples of newborns suggesting infants during development may expose to the risk of 4-t-OP contaminant, hence to investigate the effect of 4-t-OP on physiological function during embryonic development is necessary. In the present study, zebrafish embryos exposed to 4-t-OP were used to evaluate the toxicity of 4-t-OP. The 50% lethal dose (LD50) for wild type zebrafish embryos exposure to 4-t-OP for 3 days is approximately 1.0 µM, and a high ratio of cardiovascular defects were showed in survival embryos. To observe the cardiovascular defects more efficiently, Tg (fli-1: EGFP) zebrafish embryos was used in 4-t-OP exposure treatment. Following exposure Tg (fli-1: EGFP) zebrafish embryos to 4-t-OP at 1.0 µM for 4 days, a highly proportion of defects revealed in cardiovascular system, including pericardial edema, irregular shape or incomplete looping of ventricle and atrium, the absence of intersegmental vessel in the tail of notochord, unformed parachordal vessel and kinks in the caudal vein. The phenotype of cardiovascular defects was accompanied by reduced heart rate and impaired blood circulation. The mRNA expression levels of transcription factors, which are critical for zebrafish heart chamber formation and blood vessel development, were analyzed by RT-PCR. The results showed that the presence of 4-t-OP significantly induce expression level of ERα and ERβ2, and caused cardiovascular defects by suppressing transcription factor Nlx2.7, Hand2, Tbx2a, Tbx2b, Tbx5a, FGFla, GATA-4, -5 and -6 in zebrafish. The present study suggests that 4-t-OP affects the cardiovascular development in zebrafish and elucidated that early life exposure to 4-t-OP potentially may take a risk of impaired cardiovascular function.

Keywords: 4-tert-octylphenol; Toxicity; Zebrafish; Cardiovascular development

Introduction

Accompanying with the progress of human activity, loads of industrial or agricultural chemicals introduced into the aquatic environment and have been found to elicit adverse health effects in human and wildlife. One kind of these chemicals is called endocrine disrupting chemicals (EDCs) due to its interfered effects on a physiological function by mimicking or antagonizing the action of the natural hormone. The presence of environmental EDCs in animals body may alter reproduction, secretion, transport, binding and action of natural hormones that are responsible for maintenance of homeostasis [1-4]. Alkylphenol polyethoxylates (APEOs) are considered as one sort of EDCs, which belong to the group of nonionic surfactants and are widely used in the manufacturing of detergent, plastics, cosmetics, paint, and agrochemicals [5,6]. Alkylphenolic contaminants, 4-tert-octylphenol (4-t-OP), are one of microbial degradation products of APEO’s and predominant existed in various mediums of water environment, such as sewage sludge, sediments and waste water treatment plants. Several investigations have reported that 4-t-OP contamination was occurred in rivers and estuaries of Asian, European, Australia, Africa and South American rivers [7-13]. However, although several reports showed that limited level of 4-t-OP was detected in the worldwide, the presence of 4-t-OP in aquatic environment was potentially suggesting the release of 4-t-OP from industrial activities. The releasing of 4-t-OP into aquatic environment from manufacturing industries increase the probability of living organism exposure to 4-t-OP and led to the bioaccumulation of 4-t-OP in living organism through direct or indirect uptake process. In recent year global concern regarding 4-t-OP contamination in the environment potentially resulted in toxicity and damage to health due to its xenoestrogen role to disrupt endocrine function through competitive binding to the nature estrogen receptors, consequently, investigators using diverse animal model to evaluate the effects of 4-t-OP on live organism.

Studies have shown that the harmful effects of 4-t-OP on reproductive function and endocrine action of diverse fish species, for examples, the adult female of zebrafish exposed to 4-t-OP higher than 25 µg/L for 3 weeks resulted in declined ovary somatic index (OSI) in zebrafish [14]; a regress in testicular growth and vitellogenin (VTG) level induction was observed respectively in male and juvenile rainbow trout after a 3-week exposure to 30 µg/L 4-t-OP [15,16]; exposure to...
4-t-OP induced VTG synthesis and disrupts testis morphology in South American freshwater fish (*Cichlasoma dimerus*) [17]; diet supplementation of 4-t-OP in *Sparus aurata* induced alteration of liver morphology and degeneration and mediated induction of heat shock protein 70 (Hsp 70) and cathepsin genes, which are biomarkers of endocrine disruption [18]. In addition to fish model, 4-t-OP also has been proved to alter cyp19a1 expression profiles involving in gonadal differentiation of male American bullfrog [19], and susceptible to vascular function and led to the reduction of vascular contractile in rats [20]. Due to the toxicity of 4-t-OP and harmful effects over diverse species, many countries including European Union members have legislated to restrict the use of APEOs in domestic application. However, in spite of that, human still have many other pathways exposure to 4-t-OP. Recently, clinical reports showed that 4-t-OP was detected in urine samples from a 57.4% population of the 2517 subjects, and the concentration range of 4-t-OP in subjects is between 0.29g/mL and 20.6ng/mL [21]. This result potentially indicated that human already have exhibited high risk exposure to 4-t-OP from living environment including drinking water or food. This view point also can be supported by reports which showed that 4-t-OP was detected in human milk samples which is the main nourishment for newborn, and correlated finding with dietary factors [22,23]. The presence of 4-t-OP contaminant in human milk may increase health risks in newborn or infant. Moreover, report also showed that 4-t-OP was detected in 31 samples in concentrations from < 0.05 to 1.15ng/ml from 180 umbilical cord blood samples of newborns, suggesting that expectant mothers exposed to 4-t-OP and leading to contamination of fetus through blood delivery [24]. It is widely believed that embryos and infants during development are highly sensitive to chemicals that cause serious damage to development and growth; however the effect of 4-t-OP on embryonic development and physiological function of fetus so far is still unclear.

Zebrafish possess discrete organs and tissue that are similar to their human counterparts at the anatomical, physiological, and molecular level. It has become a common experimental model for studying developmental toxicity due to the advantage of rapid development, transparent body for observation; most genes have been characterized of 4-t-OP in subjects is between 0.2ng/mL and 20.6ng/mL [21]. This result potentially indicated that human already have exhibited high risk exposure to 4-t-OP from living environment including drinking water or food. This view point also can be supported by reports which showed that 4-t-OP was detected in human milk samples which is the main nourishment for newborn, and correlated finding with dietary factors [22,23]. The presence of 4-t-OP contaminant in human milk may increase health risks in newborn or infant. Moreover, report also showed that 4-t-OP was detected in 31 samples in concentrations from < 0.05 to 1.15ng/ml from 180 umbilical cord blood samples of newborns, suggesting that expectant mothers exposed to 4-t-OP and leading to contamination of fetus through blood delivery [24]. It is widely believed that embryos and infants during development are highly sensitive to chemicals that cause serious damage to development and growth; however the effect of 4-t-OP on embryonic development and physiological function of fetus so far is still unclear.

**Materials and Methods**

**Experimental animals and compound**

Adult AB-strain zebrafish and transgenic zebrafish Tg (fil-1: EGFP) were acquired from the Taiwan Zebrafish Core Facility at Academia Sinica (Taipei, Taiwan). The fish were acclimated in the laboratory culture condition and observed for clinical health for at least one week prior to experiments. The fish were raised in 10-L tanks and maintained at 28°C in recirculating freshwater with a controlled light cycle (14 h light/10 h dark), and fed daily with commercial pellet. A pair-wise breeding instead of group-breeding was used for breeding of zebrafish in this study to have a better interpretation of the effects. Fertilized embryos generated by pair-wise breeding were used for immersion treatment of 4-t-OP. All embryos were handled in compliance with the local animal welfare regulations. The alkyphenol 4-t-OP with 97% purity (CAS No. 140-66-9) was purchased from Sigma-Aldrich. The 4-t-OP is absolute ethanol as 6mM stock solution and then diluted in embryos medium for immersion treatment of zebrafish embryos. Immersion experimental design

Gastrulation is a key event during embryonic morphogenesis and therefore zebrafish embryos with gastrulation stage (5 hour post-fertilization) were used for our exposure studies. Wild type zebrafish embryos at 5 hours post-fertilization (hpf) were collected and put in 12-well microplate for immersion treatment of 4-t-OP. One hundred embryos in each well were immersed with 3 ml of embryos medium (14 mM NaCl, 0.54 mM KCl, 0.026 mM Na₂HPO₄, 0.3 mM K₂HPO₄, 0.1mM CaCl₂ and 0.1 mM MgSO₄·7H₂O in deionized water) containing 0.2 mM 1-phenyl-2-thiourea (PTU) and a various concentration of 4-t-OP, and then incubated at 28°C for 67 h. PTU added in embryos medium was used to prevent pigmentation. Embryos immersed with embryos medium containing 0.2 mM PTU was used as control group. The embryo medium was renewed daily to maintain the water quality and 4-t-OP concentration. Survival rate, hatching rate and malformation were evaluated at 3 days post-fertilization (dpf). The experiment was performed in triplicate for each condition and repeated by three times.

**Heart rate determination and morphological analysis**

Tg (fil-1:EGFP) zebrafish embryos, which enhanced green fluorescent protein (EGFP) was specifically expressed in heart and blood vessel, were used to evaluate the phenotypes of cardiovascular defects resulting from 4-t-OP treatment. Immersion treatment of Tg (fil-1: EGFP) zebrafish embryos with 4-t-OP were carried out as follows: One hundred embryos in each well of 12-well microplate was exposed to 0.5 μM or 1.0 μM of 4-t-OP from 5 hpf until the end of embryogenesis (96 hpf). The experiment was performed in triplicate. Twenty Tg (fil-1:EGFP) zebrafish embryos were picked into a petri dish containing 15ml of embryos medium at 48, 72 and 96 hpf, and heart rate of each zebrafish embryos were calculated under microscopy (Leica Z16 APO). Ten embryos were collected at 48 and 72 hpf for real-time PCR. To observe the morphological defects of heart and blood vessel, live control and 4-t-OP treated embryos were anesthetized with tricaine methanesulfonate (MS222) before mounting in 3% methyl-cellulose (Sigma M-0387) and examined under a Leica stereomicroscope. Digital images or video was acquired using a Leica camera (Leica DFC310 FX).

**Gene expression detected by real-time PCR**

The total RNA was isolated from the Tg (fil-1: EGFP) zebrafish embryos with or without 4-t-OP treatment (control group). The expression levels of estrogen receptor (ER) α, ERβ1, ERβ2, NK2 homeobox 5 (Nkx2.5), Nkx2.7, heart and neural crest derivatives expressed 2 (*Hand2*), GATA-binding protein 4 (GATA-4), GATA-5, GATA-6, fibroblast growth factor 1a (*FGF1a*), T-box 2a (*Tbx2a*), *Tbx2b, Tbx5a* and elongation factor 1-a (*ef1-a*) were determined using quantitative PCR. The *ef1-a* was used as an internal control. The specific PCR primers used in this study are listed in Table 1. Real-time PCR was performed using SYBR Green PCR reagents and an Applied Biosystems StepOnePlus Real-Time PCR system. The cycling profile was as follows: 60°C for 2 min, 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s, and annealing and primer extension at 60°C for 1 min. Equal quantities of total RNA was examined in triplicate for each condition. The relative expression levels of each group were normalized to *ef1-a* and expressed as the mean ± S.E. Student’s t-test was used to statistically analyze and compare two groups. Multiple-group comparisons were analyzed for significant differences between group using one-way ANOVA with a Tukey test (Statistica version 5.1; StatSoft, Inc., USA). The differences were defined as significant at p<0.05.
Results

Developmental toxicity of 4-tert-octylphenol

To evaluate the toxic effects of 4-t-OP on zebrafish embryogenesis, embryos were exposed to 0.1 µM, 0.5 µM, 1 µM, 2.5 µM and 5 µM 4-t-OP and compared with their corresponding control group (embryo medium contain PTU only). The survival rate, hatching rate and malformation rate exhibit dose effects to 4-t-OP treatment (Table 2).

The phenotypes of cardiovascular defect induced by 4-t-OP urge us to investigate the effect of 4-t-OP on cardiovascular function. Heart was used to characterize the cardiovascular phenotype by exposing them continuously from 5 hpf to 96 hpf.

4-tert-octylphenol induced cardiovascular defects

To easily observe the cardiovascular defect, Tg (fil-1: EGFP) zebrafish embryos were used for immersion administration. As result shown in Table 3, exposure of Tg (fil-1: EGFP) zebrafish embryos to 4-t-OP resulted in visible cardiovascular defects from 24 hpf. The cardiovascular development was normal in the control group (Figure 1A,1C,1D), however the severity of the cardiovascular defects were not consistent among 4-t-OP treated embryos. The majority of embryos exhibited pericardial edema (56% at 2 dpf) and irregular shape or incomplete looping of ventricle and atrium (28% at 2 dpf) (Figure 1B). The proportion of these phenotypes was increased following 4-t-OP exposure for 3 days, and then declined at 4 days due to the increased mortality. At certain region in the notochord tail, the absence of intersegmental vessel caused the change of the distance between intersegmental vessels and it was also exhibited in 4-t-OP treated embryos (Figure 1C). Furthermore, unfurled parachordal vessel and kinks in the caudal vein resulted in blockage of blood flow were exhibited in the 4-t-OP treated embryos (Figure 1F). These phenotypes were categorized as abnormal blood vessel development, and the proportion of these phenotypes was increased accompanying with the time of 4-t-OP treatment (Table 2).

4-t-octylphenol damage cardiovascular function

Table 1: Primer sequences and gene names.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>PCR (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor α (ERα)</td>
<td>F: CCGGCGCTCCAGAAGATGCA</td>
<td>150</td>
<td>NM_152959</td>
</tr>
<tr>
<td></td>
<td>R: ACGCAAGGCTCTCCAAACACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor β1 (ERβ1)</td>
<td>F: CTTGGCCGCTTCTGCAGATGTT</td>
<td>150</td>
<td>AF516874</td>
</tr>
<tr>
<td></td>
<td>R: CGGCGGTTCTTGGCTGATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor β2 (ERβ2)</td>
<td>F: TTTCGGGCGCTTTGCTCTGCT</td>
<td>150</td>
<td>AF349413</td>
</tr>
<tr>
<td></td>
<td>R: CGGGATGGTAAACCGCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK2 homebox 5 (Nkx2.5)</td>
<td>F: AGCTACATTCACACAGGATCA</td>
<td>150</td>
<td>NM_131421</td>
</tr>
<tr>
<td></td>
<td>R: GAGCTCCGAGGCTCGGGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK2 homebox 7 (Nkx2.7)</td>
<td>F: TGTCAAGAAGAACCCTCTCT</td>
<td>150</td>
<td>NM_131626</td>
</tr>
<tr>
<td></td>
<td>R: CCCGGTACTCTGCCGATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-binding protein 4</td>
<td>F: CAAGTCGACGGAGGATGTT</td>
<td>150</td>
<td>NM_131236</td>
</tr>
<tr>
<td></td>
<td>R: GATCGCCGACTGACCTCAG</td>
<td></td>
<td></td>
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<tr>
<td>GATA-binding protein 5</td>
<td>F: GGAGCAGCAGGAACTCTAA</td>
<td>150</td>
<td>NM_131235</td>
</tr>
<tr>
<td></td>
<td>R: CACCCGGTGACCAAGGTTG</td>
<td></td>
<td></td>
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<tr>
<td>GATA-binding protein 6</td>
<td>F: AGTCGGCAGCAGTACTTCTCA</td>
<td>150</td>
<td>NM_131557</td>
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<tr>
<td></td>
<td>R: CTTTCCAGGTTGCGAGTGT</td>
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<tr>
<td>Fibroblast growth factor 1a (FGF1a)</td>
<td>F: ATGGCAAGCTGGTACGGTCCA</td>
<td>150</td>
<td>NM_200760</td>
</tr>
<tr>
<td></td>
<td>R: GGCCGGTGTATTCTTCC</td>
<td></td>
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<tr>
<td>T-box 2a (Tbx2a)</td>
<td>F: AGTTTTCCTGTCAAGGCGATT</td>
<td>150</td>
<td>AF179405</td>
</tr>
<tr>
<td></td>
<td>R: RAGGAAAGGTGCACTGTTTCC</td>
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<tr>
<td>T-box 2c (Tbx2c)</td>
<td>F: AGTTTTTCCCTGTAGGCGATT</td>
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<td></td>
<td>R: RAGGAAAGGTGCACTGTTTCC</td>
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<tr>
<td>T-box 2a (Tbx5a)</td>
<td>F: CGGGATGGTAAACCGCTGTC</td>
<td>150</td>
<td>NM_130915</td>
</tr>
<tr>
<td></td>
<td>R: CAGCCCGTGACCAAGGTTG</td>
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<tr>
<td>Elongation factor 1a (ef1a)</td>
<td>F: GTGTTGTCGTCGGTGAGGTTG</td>
<td>150</td>
<td>AY422992</td>
</tr>
<tr>
<td></td>
<td>R: AAACGGCGTGGTGGTGAAGG</td>
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</table>

Table 2: survival rate, hatching rate and malformation rate of zebrafish embryos exposed to different concentration of 4-t-OP at 3 dpf.

<table>
<thead>
<tr>
<th>4-t-OP conc. (mM)</th>
<th>Survival rate (%)</th>
<th>Hatching rate (%)</th>
<th>Malformation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>96 ± 2.1%</td>
<td>96 ± 2.9%</td>
<td>0%</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>95 ± 1.8%</td>
<td>95 ± 1.1%</td>
<td>0%</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>79 ± 1.5%</td>
<td>83 ± 1.7%</td>
<td>28 ± 1.6%</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>52 ± 1.8%</td>
<td>71 ± 2.8%</td>
<td>60 ± 1.3%</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>26 ± 2.8%</td>
<td>45 ± 2.3%</td>
<td>87 ± 4.4%</td>
</tr>
</tbody>
</table>

*Data was evaluated from three times experiment and each experiment was triplicated (n=100).

Control indicated the embryo medium only containing 0.2% PTU.

Table 3: Cardiovascular defects induced by 4t-OP.
were significantly declined in 4-t-OP exposed zebrafish embryos at 48 and 72 hpf. The mRNA expression level of Hand2, Tbx5a, FGF1a and GATAs families including GATA-4, -5 and -6 were significantly suppressed in 4-t-OP treated zebrafish at 48 and 72 hpf. The expression level of Tbx2a and Tbx2b are significantly suppressed only in the presence of 1 µM 4-OP at 48 hpf, and significantly suppressed in the presence of 0.5 µM and 1 µM 4-OP at 72 hpf (Figure 4). These results suggested that 4-t-OP suppresses the expression level of cardiovascular development-related genes during zebrafish embryogenesis.

Discussion

Alkylphenol polyethoxylate (APEs) as nonionic surfactants has been widely used in a variety of industrial and surfactant applications. However, several investigations have reported that the unstable property of APEs in environment cause rapid degradation to hydrophobic and more toxic alkylphenols including 4-nonylphenol (4-NP) and 4-t-OP. Based on hematological and biochemical parameters examined, the study elucidated that OP had a relatively greater effect than NP and affected hematological enzymes leading to serious impairment of the metabolism and physiology in African sharp-tooth catfish (C. gariepinus) [25]. Other study reported that zebrafish embryos exposed to 1 µM of 4-OP developed normally [26], however our results showed that zebrafish exposure to 1 µM of 4-OP resulted in cardiovascular defect. These results also suggest that the toxicity effect of 4-t-OP was higher than 4-NP, and affect blood circulation of fish. In the present study the developmental toxicity of 4-t-OP on zebrafish embryos was first demonstrated that 4-t-OP disrupts zebrafish cardiovascular system. 4-t-OP exposure at 1 µM significantly decreased heart rate in zebrafish hatchlings. 4-t-OP and other endocrine-disrupting compounds have been linked to endocrine disruption mediated via interference with the estrogen and thyroid hormone systems [27]. A strong positive correlation between levels of thyroid hormone and heart rate has been demonstrated [28]. Thus, based on those studies, we assume that 4-t-OP may reduce heart rate through its effects on reducing thyroid hormone.

Transgenic biosensor zebrafish embryos which express the green florescent protein (GFP) under the control of estrogen-inducible promoter had been developed for studying potential health effects of...
ERβ1 and ERβ2 were analyzed. The presence of 4-t-OP at 0.5 µM and 1 µM significantly induced ERα, ERβ1 and ERβ2 expression in zebrafish, and higher induction level was revealed in ERα and ERβ2. Reports have showed that ERs expression can be induced by diverse estrogens or estrogen analog and different type of ER have a different binding affinity for the different ligands. Using HELN assay, whichERE-driven fulplenlength zebrafish ERα, ERβ1 and ERβ2 expression in HELa cells, 4-t-OP has been demonstrated to have greater affinity towards zebrafish ERα and ERβ2 relative to ERβ1 [33]. Our study present higher expression level induced by 4-t-OP in ERα and ERβ2 also potentially suggesting that zebrafish ERα and ERβ2 have higher affinity for 4-t-OP.

The heart is the first organ to form and function during embryogenesis and its circulatory function are critical for the viability of zebrafish embryos. The presence of 4-t-OP in zebrafish cause cardiovascular defects including incomplete looping of ventricle and atrium, defects in formation of intersegmental vessels and organization of caudal vein, and these indicators signifying that the heart development and circulation function were injured. Several genes encoding transcription factors are required for normal heart and blood vessel development. The *timen* gene encodes a NK-class of homeobox transcription factor which plays key roles in the establishment of myogenic lineages. In zebrafish, Nkx2.5 and Nkx2.7 are expressed in heart field of lateral plate mesoderm and required for cardiac morphogenesis [34]. Report has showed that morpholino (MO) knockdown Nkx2.5 in zebrafish did not affect heart development. Furthermore Nkx2.7 has been demonstrated to play a critical function in the lateral development of the heart and normal cardiac looping and chamber formation [35]. The *hand2* gene encodes bHLH1 transcription factor that regulate differentiation and the morphogenesis of the myocardial cells and involved in cardiac chamber formation. In the present study, the expression of Nkx2.7 and *hand2* is significantly declined in 4-t-OP exposed zebrafish at 48 and 72 hpf; however expression of Nkx2.5 without significant difference. This result potentially indicated the 4-t-OP induced incomplete looping of ventricle and atrium, and chamber shape through suppressing Nkx2.7 and *hand2* expression. GATA family act important transcription factors for the development of diverse tissues. *Tbx2* encodes T box factor is required for regulating heart chamber development. Report has demonstrated that two genes, *tbx5a* and *tbx5b*, were retained in zebrafish and both are required for the development of atrioventricular canal (AVC) [36]. Study also report that homozygous mutation of *tbx5a* gene in zebrafish leads to defects in cardiac looping morphogenesis [37]. The three members of GATA family, transcription factor GATA-4, -5, and GATA-6 play a critical role for heart development. GATA-5 is specifically expressed in endocardium and GATA-4 and -6 are present in the myocardium. GATA-5 and GATA-6 involved in regulating endocardial and myocardial cell differentiation [38]. GATA-4 is required for heart tube formation and ventral morphogenesis [39]. In the present study, the expression of *tbx2a*, *tbx2b*, *tbx5a*, GATA-4, -5, and GATA-6 is significantly declined in zebrafish exposure to 4-t-OP at 48 and 72 hpf suggesting that 4-t-OP suppresses the expression of these critical transcription factors and leads to defects in development and morphogenesis of heart chamber formation. Fibroblast growth factors (FGFs) are considered as important angiogenic factors for vascular development [40]. Other investigators have demonstrated that FGF signaling affects vascular outgrowth and are required for the maintenance of blood vessel integrity in zebrafish [41]. In addition to FGF, GATA-4 has been demonstrated to regulate development of the caudal vascular plexus in zebrafish through the chemokine sdf1a mediation [42]. The present result showed that downregulation of *Egf* and GATA-4 expression in the presence of 4-t-OP suggesting 4-t-OP may suppress *Egf* and GATA-4 expressions in zebrafish resulting in...
the absence of intersegmental vessel and parachorial vessel and links in the caudal vein.

In conclusion, the present study is the first report representing that the exposure of zebrafish embryos to 4-t-octylphenol resulting in highly incidence of cardiovascular defects. The presence of 4-t-OP in zebrafish embryos that induced expression level of ERα and ERβ2 suggesting the 4-t-OP mimicking estrogen which act highly binding affinity with both ER. The 4-t-OP exposed zebrafish embryos resulted in suppression of transcription factor \( \text{NKX2.7}, \ \text{hand2}, \ \text{Tbx2a}, \ \text{Tbx2b}, \ \text{Tbx5a}, \ \text{FGF1a}, \ \text{GATA-4}, \ -5 \) and \(-6\) expression may be the cause of cardiovascular defects. The susceptibility of zebrafish model exposed to 4-t-OP during early life suggests its role in injuring cardiovascular development and function, which is a health-risk concern of early life exposure in humans.

**Acknowledgment**

We thanks the Taiwan Zebrafish Core Facility at Academia Sinica (TZACS), which is supported by grant NSC 103-2321-B-001-050 from the Ministry of Science and Technology (MOST) in Taiwan for providing AB strain zebrafish and transgenic zebrafish \( \text{Tg(fil-1:EGFP)} \). This research was supported by a grant NSC 102-2313-B-02-015 from the Ministry of Science and Technology.

Figure 4: Relative expression level of selected genes in 4-t-OP exposed \( \text{Tg(fil-1: EGFP)} \) zebrafish embryos were determined by real time PCR at 48 and 72 hpf. The expressions of the cardiovascular-related genes \( \text{Nkx2.5, Nkx2.7, Hand2, Tbx2a, Tbx2b, Tbx5a, FGF1a, GATA-4, -5 and -6} \) were quantified. The data of the control group at 48 hpf was set as 1. The results represented triplicated experiments, and the different letters were considered significantly at \( p<0.05 \).
References


35. Tu CT, Yang TC, Tsai HJ (2009) Nkox2.7 and Nkox2.5 function redundantly and are required for cardiac morphogenesis of zebrafish embryos. PLoS One 4: e4249.


