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Effects of triclosan on antioxidant- and apoptosis-related genes expression in the gill and ovary of zebrafish

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Abstract: Triclosan (TCS) is a broad-spectrum antibacterial and anti-fungal agent used in a broad variety of personal care products (PCPs) throughout the world. However, the molecular mechanism of TCS's effects on the gill and ovary of fish is not clear. In this study, the effects of TCS exposure on expression of antioxidant- and apoptosis-related genes were investigated in the gill and ovary of zebrafish (*Danio rerio*). Zebrafish were exposed to 0, 17, 34, or 68 μg/l TCS for 42 days. Antioxidant-related genes (*SOD*, *GPx1a*, *CAT*, *sMT-B*, and *MT-2*) in the gill were significantly downregulated in the 34 (except *GPx1a*) and 68 μg/l TCS groups, and these genes (except *MT-2*) in the ovary were significantly downregulated in the 68 μg/l TCS group. Apoptosis-related gene (*Bax* and *p53*) expression level in the gill were significantly downregulated in the 68 μg/l TCS group, while the ratios of *BCL-2* to *Bax* and *MDM2* gene were significantly upregulated. The *Bax* gene in the ovary was significantly upregulated in the 34 and 68 μg/l TCS groups, while the ratio of *BCL-2* to *Bax* was significantly downregulated. Moreover, the *p53* gene in the ovary in the 34 μg/l TCS group was significantly upregulated. In addition, the MDA contents in the gill in the 34 and 68 μg/l TCS treated groups and in the ovary in 68 μg/l group were significantly increased. The results showed that the higher dose of TCS might cause oxidative damage in the gills and ovaries and accelerate ROS-dependent ovary apoptosis in zebrafish.
Key words: antioxidation, apoptosis, gill, ovary, triclosan

Introduction

Triclosan (TCS) is an efficient, lipophilic synthetic broad-spectrum antibacterial and anti-fungal agent used in a broad variety of personal care products (PCPs), including toothpaste, soaps, shampoos, and other sanitation goods [9, 40]. Currently, widespread pollution with TCS has been detected in aquatic ecosystems of many countries [9]. TCS has been found in sewage treatment plant effluent in quantities up to micromolar (μg/l) concentrations in North America, Europe, and Asia [24, 25, 31, 32, 47]. Although most TCS is removed during sewage treatment, a survey of over 100 streams in the United States (USA) indicated that TCS was present in 57.6% of sites examined, with the maximum concentration being 2.3 μg/l [24]. Similar levels of TCS are also found in many rivers and lakes of Europe, Oceania, and Asia [9]. TCS or its derivative, methyl-triclosan, may bioaccumulate in animal tissues, and it has also been found in the urine and breast milk of people [16]. Therefore, TCS is considered to be the priority control pollutant by the European Union, USA, and China and to be a carcinogen by the International Agency for Research on Cancer (IARC) [41, 46].

TCS has effects on nontarget organisms. It is reported that exposure to TCS in the environment causes biological genotoxicity, developmental and reproductive toxicity, hepatotoxicity, endocrine toxicity, immunotoxicity, neurotoxicity, cardiotoxicity, and carcinogenesis effects [49]. Results obtained in the freshwater mussel *Dreissena polymorpha* by using *in vitro* and *in vivo* experiments offered evidence that TCS caused oxidative

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stress, which was possibly one of the main toxicity mechanisms of TCS [4, 5, 36].

Oxidative stress has become an important theme in aquatic toxicology in recent years, and more and more attention has been paid to the mechanism of oxidative damage and cellular response in biological systems [22]. Reactive oxygen species (ROS), such as superoxide anion radicals ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH), are continuously produced in oxygen-consuming organisms. Exposure to toxic chemical pollutants may lead to an imbalance between these endogenous and exogenous ROS and can subsequently cause a decrease in antioxidant defenses or lead to oxidative damage in organisms [42].

Vertebrates try to decrease the damage from oxidative stress using an antioxidant defense system which includes antioxidant molecules (such as glutathione[GSH] and vitamins C and E), antioxidant enzymes (such as superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx]), and other antioxidant proteins (such as, metallothionein-like protein). Recently, measurements of antioxidative enzyme activities in fish have been used to assess the oxidative damage caused by toxic chemical pollutants in aquatic ecosystems [7, 17, 20]. Correspondingly, several studies have shown that TCS can cause oxidative stress by increasing the level of ROS and products of oxidative damage (such as lipid peroxides) and by affecting antioxidant enzyme activities in some organisms [11, 35]. It is reported that TCS exposure could cause an increase in ROS content (%) and glutathione transferase (GST) enzymatic activity in the monogonont rotifer (*Brachionus koreanus*) [15]. More recently, our previous study also confirmed that TCS could increase the antioxidant enzyme of CAT and the GSH contents and that it even induced excessive ROS production and DNA damage in the liver of goldfish after 14 d of exposure. These findings suggest that subchronic TCS concentrations can lead to oxidative damage in goldfish (*Carassius auratus*) [43].

Anti-oxidants such as N-acetylcysteine (NAC) can hinder apoptosis [29], suggesting that excessive oxidative stress may induce cell apoptosis. Apoptosis can be regulated by specific proteins which produce pro-apoptotic and anti-apoptotic signals. Bcl-2 family proteins are anti-apoptotic proteins [48]. Bax can promote apoptosis of cells. Bcl-2 can interact with bax protein to regulate the onset apoptosis [19]. P53 can upregulate the expression of Bax and downregulate the expression of Bcl-2 to promote apoptosis [37]. It can also induce apoptosis through the death signal receptor protein pathway. MDM2 is p53's negative regulator [10]. These findings show that the molecular mechanism of apoptosis is extremely complex.

The zebrafish (*Danio rerio*) is an *in vivo* experimental animal model. The gill is usually one of the main target organs of fish for toxic effects of toxic chemicals [50]. There are reports of reproductive health effects related to TCS [21], and our previous experiments confirmed that TCS had an interference effect on the reproductive endocrine system in female Yellow River carp (*Cyprinus carpio*) [44]. Gene transcription level changes would cause earlier detection and measurement of toxicant effects [12]. Therefore, in the present study, the effects of environmental-related concentrations of TCS exposure on expression of antioxidant- and apoptosis-related genes were investigated in gill and ovary of zebrafish. In addition, we also examined malondialdehyde (MDA), a marker of oxidative stress, in the gill and ovary. This information is intended to provide new insights into the toxicological mechanism of TCS.

Materials and Methods

Test chemicals

TCS (CAS: 3380-34-5; 99.8%) was purchased from Jiangsu Equalchem Co., Ltd (Jiangsu, China), and stock solutions were prepared by dissolving the powder in dimethyl sulfoxide (DMSO; Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China) at a concentration of 2,000 mg/l. The stock solutions were used to prepare TCS treatment solutions via dilution to the required concentrations in the present study. DMSO in the test solution was kept in 0.07%.

Experimental fish

All animal use was approved by School of Life Sciences, Luoyang Normal University Institutional Animal Care Committee and complied with the Institutional Guidelines for the Care and Use of Laboratory Animals. Wild-type (AB strain) zebrafish (*Danio rerio*) were obtained from China Zebrafish Resource Center (CZRC) at 7 weeks old. The mean body weights and lengths were 0.06 ± 0.01 g and 1.62± 0.31 cm. Zebrafish were maintained in 60-l glass tanks containing 30 l aerated tap water at 26 ± 2°C under a photoperiod 14:10 h light/dark in the Ecotoxicology Laboratory at Luoyang Normal University (Luoyang, China). The fish were fed twice a day with *Artemia* nauplii *ad libitum*. After feeding, the remaining food and feces were removed within 30 min. At the end of 2 weeks of acclimation, the zebrafish TCS exposure experiment began.

TCS exposure

Zebrafish were continuously exposed to 0 (control),

17, 34, or 68 μg/l TCS for 42 d. Experimental concentrations were chosen based on the LC_{50} (340 μg/l) at 96 h and the environmental concentration of TCS for zebrafish [34]. Two hundred and forty fish (twenty fish/tank, three replicates) were used for each control or TCS treatment group. The TCS test solution in each tank was replaced with half-fresh TCS test solution every day. The other conditions were in accordance with those during the acclimation period.

TCS concentrations in tested solutions were measured by high-performance liquid chromatography with ultraviolet detection. Detailed procedures for analysis of tank TCS concentrations were conducted as described in our previously established protocols [44]. Based on analysis of the test solutions, the TCS concentrations in the TCS-treated tanks during the 42-day exposure experiment were as follows: 13.5 ± 3.4, 28.3 ± 6.7, and 56.4 ± 9.2 μg/l, respectively. The TCS concentration in the control group was lower than the lower limit of detection.

The gills and ovaries excised from zebrafish in each exposure tank were divided into one sample of each was collected from three zebrafish in each experimental group, resulting in six samples for each experiment group. These samples were immediately snap-frozen in liquid nitrogen and then stored in an ultralow temperature refrigerator (−80°C) until use. No mortality was observed in any of the experimental groups during the whole exposure period.

Gene expression analysis

Total RNA was isolated from the pooled gills and ovaries of the zebrafish using RNAfast200 reagent (Fastagen Biotech, Shanghai, China) according to the manufacturer's instructions. The ratio of A_{260} to A_{280} was checked by ultramicro spectrophotometer and 1% agarose gel electrophoresis and used to verify the quality of the total RNA.

Subsequently, the RNA was denatured at 65°C for 5 min. cDNA was synthesized using First Strand cDNA Synthesis Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. Denatured RNA was used as the template and was reverse- transcribed to cDNA in a final reaction volume of 10.0 μl. Tt was then incubate at 37°C for 15 min and then 98°C for 5 min to inactivate the enzyme. The reversed cDNA was adjusted to the appropriate concentration and stored in a refrigerator.

Real-time quantitative PCR (RT-qPCR) was performed using a SYBR Green PCR kit (DBI® Bioscience, Ludwigshafen, Germany) and analyzed on an Bio-Rad CFX96 Touch Sequence Detector System. Specific primers were used to detect the gene expression of *β-actin*, *SOD*, *GPx1a*, *CAT*, *sMT-B*, *MT-2*, *Bcl-2*, *Bax*, *p53*, and *MDM2*, and the detailed information is shown for them in Table 1. RT-qPCR amplification was carried out in 25-µl reaction mixtures which included 10 µl Bestar SybrGreen qPCR mastermix, 1 µl first-strand cDNA (template), 0.5 µl PCR Forward Primer, and 0.5 µl PCR Reverse Primer. The reaction was performed at 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. As an internal reference gene, β-actin transcript was used to standardize the results, and each target gene mRNA level was expressed as its ratio to β-actin mRNA. The relative quantification of each target gene expression among the experiment groups was

Table 1. Sequences of primer pairs used in the real-time quantitative PCR reactions

Primer Name	Sequence (5' to 3')	Accession number	Tm (°C)
β-actin	CGGAATCCACCAAACCACCTA ATCTCCTTCTGCATCCTGTGA	NM_181601.5	56
SOD	GGCCAACCGATAGTGTTAGA CCAGCGTTGCCAGTTTTTAG	NM_131294.1	57
GPx1a	ACCTGTCCGCGAAACTATTG TGACTGTTGTGCCTCAAAGC	NM_001007281.2	58
CAT	AGGGCAACTGGGATCTTACA TTTATGGGACCAGACCTTGG	XM_021470442.1	58
sMT-B	TGCTCCAAATCTGGATCTTG GCAGTCCTTCTTGCCCTTAC	NM_001201469.1	58
MT-2	AGACTGGAACTTGCAACTGTGGT CAGCTGGAGCCACAGGAATT	NM_001131053.3	62
Bcl-2	F:ATGTGCGTGGAAAGCGTCAAC R:GAAGGCATCCCAACCTCCATT	NM_001030253.2	56
p53	F:GGGCAGGGAGCGTTATGA R:AGAGTCGCTTCTTCCTTCGTC	NM_001271820.1	56
MDM2	F:CCGACGCCTCCACTTCTC R:ATAAGGTGCCCAGTCCTTCC	AF010255.1	56
Bax	F:CGATACGGGCAGTGGCA R:TCGGCTGAAGATTAGAGTTGTT	AF231015.1	56

analyzed by the $2^{-\Delta\Delta ct}$ method.

Assay of MDA in the gill and ovary

The whole process of preparation of gill and ovary homogenates of zebrafish was performed on ice. First of all, the gills and ovaries were thawed, then were washed with a 0.86% normal saline at 4°C, dried and weighed, and put into a tissue grinder immediately with 0.01 mol/l Tris–HCl buffer solution at pH 8.0 using a mass ratio of 1:9. The homogenates were then collected in centrifuge tubes and centrifuged at 1,006 g for 20 min. The supernatants were collected to determine the levels of MDA and protein.

MDA and protein were measured by using MDA and protein determination kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). MDA in gill and ovary homogenates was determined in terms of thiobarbituric acid reactive substances (TBARSs) formation with maximal absorbance at 532 nm by following the protocol. The concentration of MDA in the gill and ovary of the zebrafish was calculated by comparing the absorbance to that produced by the control standard 1, 1, 3,3-tetraethoxypropane and expressed as nmol/mg prot.

Statistics analysis

Data analyses were conducted using the SPSS software (Ver.17.0,SPSS Inc., Chicago, IL, USA). Data were expressed as means ± SD. One-way ANOVA (Duncan's multiple comparison test) was applied for significance tests to verify differences between the control and the different TCS treatment groups.

Results

Effects of TCS on mRNA expression of oxidative stress-related genes in the gill and ovary of zebrafish

Treatment with TCS resulted in decreased oxidative stress-related mRNA levels in the gill of zebrafish (Fig. 2A). The inhibitory effects on the *SOD*, *CAT*, and *sMT-B* genes were all highly significant for 34 and 68 μg/l TCS (50.6% and 77.7%, 94.1% and 90.2% and 65.4% and 65.4%, respectively, $P<0.01$). The *GPx1a* inhibitory effect was significant for 68 μg/l TCS (58.4%, $P<0.05$). The *MT-2* inhibitory effects were highly significant for 17, 34, and 68 μg/l TCS (95.1%, 78.5%, and 56.7%, respectively, $P<0.01$).

The effects of TCS on the expression of oxidative stress-related genes in the ovary of zebrafish were shown in Fig. 1B. The inhibitory effects in *SOD*, *CAT*, *GPx1a*, and *sMT-B* genes were also highly significant or significant for 68 μg/l TCS (39.8%, 55.2%, 53.9%, and 56.5%, respectively), while the induction effects of *MT-2* were significant for 34 μg/l TCS (1.84 fold, $P<0.05$).

Effects of TCS on mRNA expression of apoptosis-related genes in the gill and ovary of zebrafish

The effects of TCS on the expression of apoptosis-related genes in the gill and ovary of zebrafish are shown in Figs. 2A and B, respectively. The *Bax* and *p53* mRNA relative expression levels in the gill in the 68 μg/l TCS group were significantly decreased compared with the control ($P<0.05$ for *Bax*; $P<0.01$ for *p53*). The inhibitory rates were 94.9% and 95.3%, respectively. However, the *Bax* gene in the ovary in the 34 and 68 µg/L TCS treated groups and *p53* in the 34 μg/l TCS treated group were significantly increased compared with the control (1.52 fold, 1.84 fold, and 3.82 fold, respectively; $P<0.05$ for *Bax* ; $P<0.01$ for *p53*). The ratio of *BCL-2* to *Bax* in the gill in the 68 μg/l TCS treated group was significantly increased compared with the control ($P<0.01$), while the ratio of *BCL-2* to *Bax* in the ovary in the 34 and 68μg/l TCS treated groups was significantly decreased ($P<0.01$). In addition, the *MDM2* mRNA relative expression levels in the gill in the 34 and 68 μg/l TCS groups were significantly increased compared with the control (6.6 fold and 10.8 fold, respectively, $P<0.01$). The results showed that the effects of TCS on the regulation of apoptosis-related genes expression in the ovaries and gills of zebrafish are different.

Effects of TCS on MDA in the gill and ovary of zebrafish

MDA is widely used as a biomarker for assaying oxidative stress in the field of toxicology and pharmacology. In the present study, the MDA contents in the gill in the 34 and 68 μg/l TCS groups were significantly increased compared with the control, and in the ovary in the 68 μg/l TCS group, it was also significantly increased (Fig. 3). The results showed that a higher concentration of TCS caused oxidative damage to the gills and ovaries of zebrafish.

Discussion

When an organism is exposed to environmental pollutants, oxidative stress, defined as an imbalance between the production and consumption of ROS [23, 42],often occurs Recently, differential transcription levels of genes encoding stress-related proteins and antioxidant enzymes have been used to detect biological toxicity and/or to discuss the effects of chemical pollutants [1, 20, 27]. For this reason, we examined the transcription levels of representative genes, which encode proteins and antioxidant

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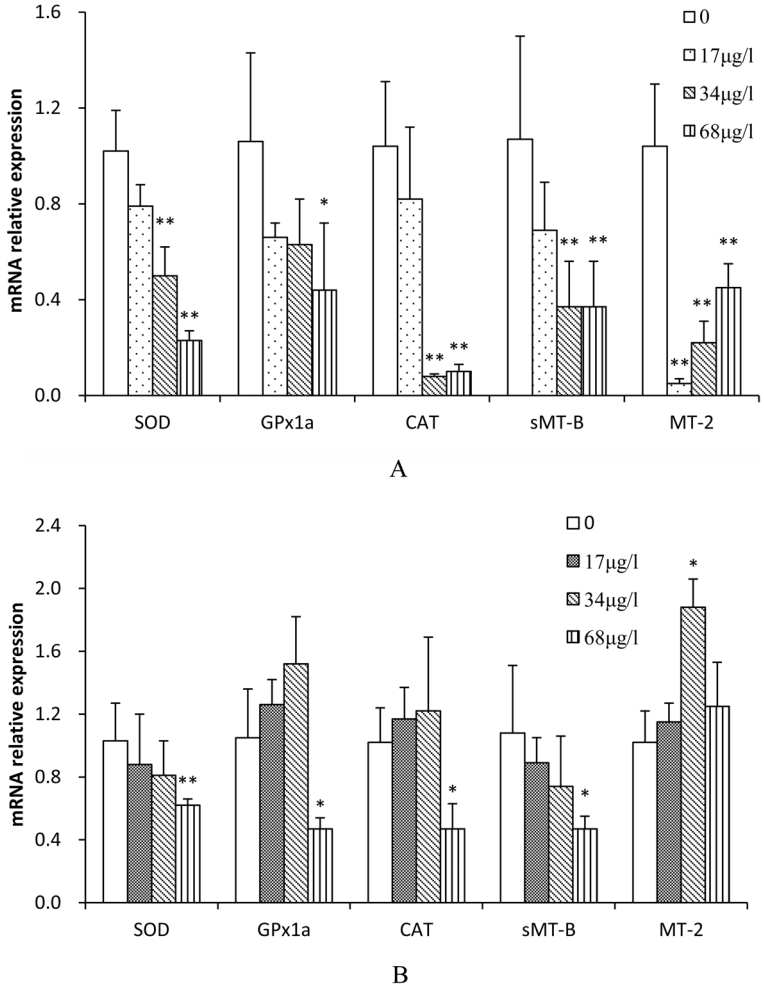

Fig. 1. Expression of antioxidant-related genes in zebrafish (A, gill; B, ovary) exposed to various concentrations of TCS for 42 d. Values were normalized against β-actin (used as a house-keeping gene) and presented as the mean mRNA expression value ± SD (n=6) relative to those of the controls. Asterisks indicate statistically significant differences when compared with the controls. * $P<0.05$; ** $P<0.01$.

enzymes that are used to resist oxidative stress. In order to confirm whether TCS causes oxidative stress in zebrafish, we also examined MDA, a marker of oxidative stress.

Antioxidant enzymes include important ROS scavenging enzymes, such as SOD, CAT, and GPx, which are the first line of defense against ROS [18]. In the present study, *SOD*, *CAT*, and *GPx* gene expression levels in the gill were significantly downregulated in the 34 (except *GPx1a*) and 68 μg/l TCS groups, and the levels of these genes in the ovary were also significantly downregulated in the 68 μg/l TCS group. These results showed that a high dose of TCS significantly affected the transcription levels of antioxidant enzymes-related genes, which may influence the expression of antioxidant enzymes. Decreased gene expression of *SOD*, *CAT*, and *GPx1a* observed in the gill and ovary at the higher doses of TCS may be a consequence of an inhibition caused directly by the xenobiotic action via ROS accumulation or via key damages in the structure of the enzymes induced by ROS [39]. The mechanism of direct inhibition of antioxidant enzymes by xenobiotics is unclear and mainly depends on the type of compound. In addition, the action time and concentration of a xenobiotic determine the amount of ROS accumulated, which could result in inhibition of SOD, CAT, and GPx, respectively [28]. Therefore, a high dose of TCS could cause accumulation of excessive ROS, resulting in decreased *SOD*, *CAT*, and *GPx* gene expression.

Metallothioneins (MTs) are thiol-rich metal-binding proteins with a wide range of functions, one of which is defense against oxidative damage [13]. Previous studies have shown that MTs have antioxidant activity and together with GSH, play an important role in regulating redox balance and scavenging superoxide and hydroxyl radicals [33]. There are many types of MTs in zebrafish. In the present study, the *MT2* gene expression levels in the gill in the TCS treated groups and the *sMT-B* gene expression levels in the 34 and 68 μg/l TCS groups were

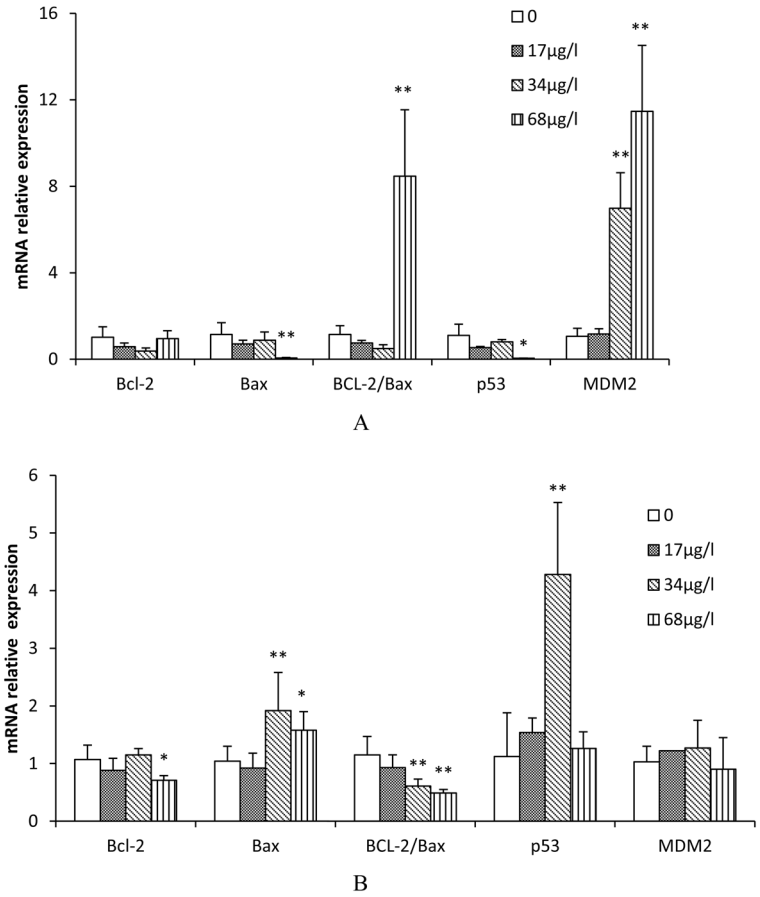

Fig. 2. Expression of apoptosis-related genes in zebrafish (A, gill; B, ovary) exposed to various concentrations of TCS for 42 d. Values were normalized against β-actin (used as a house-keeping gene) and presented as the mean mRNA expression value ± SD (n=6) relative to those of the controls. Asterisks indicate statistically significant differences when compared with the controls. * $P<0.05$; ** $P<0.01$.

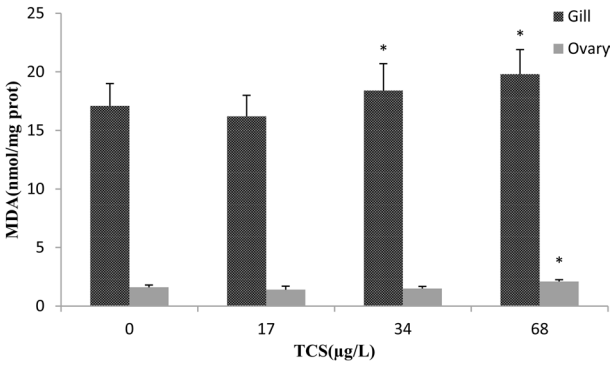

Fig. 3 . Effects of TCS on MDA in the gill and ovary of zebrafish exposed to various concentrations of TCS for 42 d. Asterisks indicate statistically significant differences from the control group (*$P<0.05$). Data are presented as the mean ± SD (n=6).

significantly decreased compared with the control, while the MDA contents were significantly increased. These results further confirm that a high dose TCS may cause oxidative damage to the gills of zebrafish. In addition, the *sMT-B* gene expression in the ovary in the 68 μg/l TCS group was significantly decreased compared with the control, while the *MT-2* gene expression in the 34 μg/l TCS group was significantly increased. The results showed that *MT-2* in the ovary in the 34μgl TCS group can be used as a sensor and amplification system for oxidative stress, in which the oxidation of *MT-2* releases zinc through thioprotein and GSH. Findings of several previous reports are in agreement with the findings in the present study [6, 45]. Therefore, it is possible that increased *MT-2* enhanced the antioxidative capacity in the ovary in the 34μg/l TCS group of zebrafish. However, the results showed that the MDA content in the ovary in the 68μg/l TCS group was significantly increased, while the decrease in *sMT-B* gene expression in the ovary in the 68μgl TCS group showed that TCS caused oxidative damage to the ovary under this condition.

Mitochondrial dysfunction following oxidative damage is one of the early events in apoptotic cell death, as the pro-apoptotic factor cytochrome C (Cyt C) is released into the cytoplasm [38]. Release of Cyt C from the mitochondria can be triggered by the pro-apoptogenic Bax [3], while the anti-apoptotic Bcl-2 can prevent the release of Cyt C [2], and Bcl-2 can interact with bax protein to

regulate the onset of apoptosis [19]. Moreover, the increased ROS production decreases the Bcl-2/Bax ratio [8]. In the present study, *Bax* gene expression in the ovary in the 68 μg/l TCS group was significantly increased, while the *Bcl-2/Bax* ratio and *Bcl-2* gene expression were significantly decreased in the 34 and 68 μg/l TCS groups. These results showed that a high dose of TCS could cause mitochondrial dysfunction to accelerate ovary apoptosis. However, in the gill, *Bcl-2* gene expression in the TCS groups was not significantly changed, whereas the *Bax* mRNA expression and *Bcl-2/Bax* ratio in the 68μg/l TCS group were significantly decreased and increased, respectively, suggesting that ROS generation was not related to apoptosis in the gill of zebrafish exposed to TCS and that a high dose of TCS inhibited gill apoptosis in our study.

ROS are involved in the signaling pathways that cause the mitochondrial migration of p53, which is involved in triggering ROS-dependent cell apoptosis [14, 26]. Elevated levels of p53 upregulate the expression of the proto-oncogene *MDM2* [10, 30]. Moreover, p53 can promote apoptosis by upregulating the expression of *Bax* and downregulating the expression of *Bcl-2* [37]. In the ovary in the present study, *p53* gene expression was significantly increased in the 34μg/l TCS group, and *MDM2* gene expression was unchanged in all TCS groups; *p53* gene expression was significantly decreased in the 68μg/lgroup, and NDM2 gene expression was significantly increased in the 34 and 68 μg/l TCS groups. These results further confirmed that a higher dose of TCS might accelerate ovary ROS-dependent apoptosis but inhibit gill apoptosis.

In conclusion, a high dose of TCS exposure downregulates significantly the expression of antioxidant-related genes, which may lead to oxidative damage to the gill and ovary of zebrafish. In addition, a higher dose of TCS might accelerate ovary ROS-dependent apoptosis. To our knowledge, this is the first report to study the oxidative stress and apoptosis-related transcription level effects of TCS on zebrafish. Thus, the information presented in the present study is helpful for understanding the mechanism of TCS-induced oxidative stress and apoptosis in fish.

Acknowledgments

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