B-4686
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RESEARCH ARTICLE Indian Journal of Animal Research

Short Term Effects of Antimicrobial Agent Triclosan on *Oreochromis mossambicus* (Peters, 1852): Biochemical and Genetic Alterations

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10.18805/IJAR.B-4686

ABSTRACT

Background: Triclosan is an antimicrobial agent which enters into the aquatic environment through wastewater discharges which causes potential health risk in human and aquatic organisms. The present study aimed to determine the toxic effects of triclosan on *Oreochromis mossambicus*.

Methods: The fishes were subjected to five different concentrations *viz*. 131, 262, 523, 1046 and 2092 µg.l^{-1} of triclosan for 96 h acute toxicity test. To evaluate the levels of enzymes such as acetylcholinesterase and glutathione S transferase, brain and liver tissues were collected, homogenized, extracted and stored at -20° for further analysis. The DNA damage was assessed in gill and liver tissues using single cell gel electrophoresis method.

Result: In present study, the calculated 96 h LC_{50} value of triclosan in *O. mossambicus* was 740 µg.l^{-1} and the fishes showed various behavioural alterations. Time and dose dependent inhibition of AChE activity in brain tissue was observed during acute toxicity test. However, the GST activity in liver tissue increased on exposure to triclosan with significant increase in concentration of toxicant. DNA damage index was higher in gill tissue compared to the liver tissue during acute exposure to TCS which could cause detrimental effects in fishes.

Key words: Acute toxicity, DNA damage, Enzyme activity, *Oreochromis mossambicus*, Triclosan.

INTRODUCTION

In the modern world, anthropogenic pollution causes potential threat to aquatic ecosystem by interference in the normal metabolism, reproduction and development of aquatic organisms. Triclosan (TCS) (CAS registration number 3380-34-5) also known as 5-chloro-2-(2,4-dichlorophenoxy) phenol or 2,4,4′-trichloro-2′-hydroxydiphenyl ether, is a broad-spectrum antimicrobial agent in some personal care products such as toothpastes, soaps, hand washes, deodorants, kitchen and plastic wares (Fang *et al*., 2010; MacIsaac *et al*. 2014). Its widespread use in homes and in health care centres is the reason that it has become a water micro-pollutant (Li *et al*. 2010; Helbing *et al*., 2011). Due to its high lipophilicity (log K_{ow} = 4.8 octanol - water partition coefficient), it has serious distress about bioaccumulation in fatty tissues which raises toxicity concern in aquatic organisms (Behera *et al*. 2011).

Toxicity study helps to understand the effects of environmental pollutants on aquatic organisms by bioassay methods (Srivastava *et al*., 2016). Biomarkers can provide biologically and ecologically relevant information and considered as a valuable tool for the establishment of guidelines for effective environmental management (Tashla *et al*., 2018). Acetylcholinesterase (AChE) is one of the most commonly used biomarker enzymes of neurotoxicity and found at neuromuscular junctions and cholinergic synapses at the central nervous system. Main role of AChE is to regulate the nerve impulse transmission by hydrolysis of the neurotransmitter acetylcholine (Lionetto *et al*. 2013). Xenobiotics entering the aquatic environment can inhibit the AChE activity in brain which can lead to the behavioural

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How to cite this article: Deepika, S., Padmavathy, P., Srinivasan, A., Sugumar, G. and Jawahar, P. (2021). Short Term Effects of Antimicrobial Agent Triclosan on *Oreochromis mossambicus* (Peters, 1852): Biochemical and Genetic Alterations. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4686.

Submitted: 22-06-2021 **Accepted:** 20-08-2021 **Online:** 24-09-2021

alteration of fish (Ghazala *et al*., 2016). Some researchers have identified the reduction of AChE activity in the brain tissue due to TCS in *Danio rerio*, *Pangasionodon hypothalamus, Cyprinus carpio, Ctenopharyngodon idella, Labeo rohita* and *Cirrhinus mrigala* which was found to be more intense during the early life stages (Oliveira *et al*. 2009; Sahu *et al*., 2018; Dar *et al*., 2020). Glutathione S-transferase (GST) is the multifunctional phase II intracellular enzyme involves in detoxification of endogenous and exogenous toxic compounds. Pollutants are known to inhibit GST activity in aquatic organisms. Elevated level of GST activity in the tissues indicates increase in the biotransformation process of toxic metabolites for excretion (Modesto and Martinez, 2010). Genotoxicity study using comet assay is a most sensitive and economical technique to detect the genetic damage at cellular level. Comet assay is a versatile and simple way of evaluating the damage caused by the pollutants by measuring the breaks in the DNA chain of animal cell (de Lapuente *et al*. 2015). Therefore, the above biochemical and genetic parameters were chosen for the present study to evaluate the acute toxicity of TCS in *Oreochromis mossambicus* which is the commercially important and candidate species for aquaculture in food security point of view.

MATERIALS AND METHODS

Experimental fish

Experimental fish *O. mossambicus* (weight: 102.75 ± 2.63g; length: 15.73±0.68 cm) was procured from the freshwater farm facility of Fisheries College and Research Institute, Thoothukudi, Tamil Nadu, India and the study was conducted during the year 2019. The fishes were acclimatized to the laboratory condition in fibreglass reinforced plastic (FRP) rectangular tank of 500 L capacity with proper water exchange and aeration. During the acclimatization the fishes were fed with commercial fish feed. The ethical guidelines for the animal care of the institute were strictly followed during the experiment.

Chemicals

Triclosan or 2, 4, 4' - Trichloro - 2' - hydroxydiphenyl ether (CAS id: 3380-34-5) was purchased from Tokyo Chemical Industry Co., Ltd. (purity >98%) and used for the present study. Stock solution of 200 ppm was prepared for the toxicity study using 0.01N NaOH solution as solvent for dissolving the TCS.

Test conditions

Water quality parameters of the water used for all the experiments *viz*. water temperature, pH, dissolved oxygen, alkalinity and hardness were analysed during the toxicity study according to standard methods of APHA (2012). Static renewal test was followed for the acute and sublethal toxicity experiments. The test solutions were totally renewed at 24 h interval during the experimental period. The tests were conducted in duplicate in plastic tanks of 50L capacity and ten fishes per tank were used for different concentrations of test chemical, solvent control (0.01N NaOH) and blank control. All experiments were conducted in indoor area with natural 12h:12h light - dark photoperiod. Before 96 h acute toxicity test, the toxicity range - finding test was conducted for 48 h by following the method of EPA (2002). Randomly selected fishes were exposed to the test concentrations in logarithmic series (*i.e.* 0.01, 0.1, 1.0, 10 and 100 mg.l^{-1}) along with solvent and blank control.

Acute toxicity test

Based on the result of range - finding test, five experimental concentrations of TCS for *O. mossambicus* (131, 262, 523, 1046 and 2092 µg.l^{-1}) was selected to conduct 96 h acute toxicity test and determined the median lethal concentration (LC_{50}) of TCS. Control group (toxicant free group) of fishes were maintained as the reference group for comparison with solvent (NaOH group) and TCS exposed fishes. During the entire test period no feed was offered to the fishes. The physico chemical parameters of the test conditions were monitored daily for 96 h of the test period. Behavioural changes and fish mortality were monitored for 96 h and the dead animals were removed from the test conditions immediately to prevent contamination. The LC_{50} values were calculated using the mortality data by probit analysis (Finney, 1971).

Collection of tissues and preparation of homogenates

After 96 h of exposure to TCS in acute toxicity test the organs such as gill, liver and brain were collected from each treatment and control group of fishes. The collected organs were homogenised with 0.25 M sucrose buffer solution (1:10 w/v). The homogenates were centrifuged at 14,000 x g for 15 min at 4p°C to obtain supernatants which were used for further enzymatic analysis. All the supernatants were stored at -20p°C until further analysis.

Enzyme assay

The activity of acetylcholinesterase (AchE; EC 3.1.1.7) was measured in brain tissue spectrophotometrically at 405 nm at 25p°C according to the method of Ellman (1961) with some modifications described by Augustinsson (1957). The activity of AChE was expressed as µM of acetylcholine hydrolyzed $min^{-1}mg^{-1}$ of protein.

The activity of glutathione S transferase (GST; EC 2.5.1.18) was measured in liver tissue at 340 nm spectrophotometrically at 25p°C according to Habig *et al.* (1974). The GST activity of liver following the formation of GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) was expressed as n mol. CDNB conjugates formed min^{-1} mg^{-1} of protein.

Single cell gel electrophoresis (comet assay)

The alkaline single cell gel electrophoresis (SCGE) / comet assay was performed in gill and liver tissues of tilapia

according to the standard procedure of Singh *et al.* (1988) with some modifications as described by Ali *et al*. (2008).

Statistical analysis

Differences in the test concentration and control were subjected to one way analysis of variance (ANOVA) followed by Turkey's multiple range test using statistical package SPSS 22.0. Difference in the mean range test and influence of TCS concentration in fishes were determined at 5% probability level. The results were presented as mean ± standard error.

RESULTS AND DISCUSSION

Determination of LC_{50} and behavioural responses

Triclosan has been classified as a class III drug (compounds with low permeability and high solubility) by FDA due to their broad spectrum antimicrobial activity (Dhillon *et al*., 2015). Toxicity studies conducted on various organisms proved that TCS is acutely and chronically toxic to all the organisms especially to the aquatic organism through wastewaters from different sources. Earlier studies observed the median lethal concentration (LC_{50}) of TCS on different fishes as 4400 $\mu g.l^{-1}$ in *Onchorynchus mykiss* (Adolfsson-Erici *et al.*, 2002), 370 and 1700 $\mu g.l^{-1}$ in *Oryzias latipes* (Orvos *et al.*, 2002 and Nassef *et al.*, 2009), 340 $\mu g.l^{-1}$ in *Danio rerio* (Oliviera *et al*, 2009), 1480 $\mu g.l^{-1}$ in *Xiphophorus helleri* (Liang *et al*., 2013), 2810 $\mu g.l^{-1}$ in *Oreochromis niloticus* (Vijitha *et al.,* 2017), 1458 $\mu g.l^{-1}$ in *Pangasianodon hypophthalmus* (Sahu *et al.*, 2018), 1767 $\mu g.l^{-1}$ in *Anabas testudineus* (Priyatha and Chitra, 2018), 360 $\mu g.l^{-1}$ in *Gibelion catla* and 390 $\mu g.l^{-1}$ in *Labeo rohita* (Hemalatha *et al.*, 2019a and 2019b). In the present study, the LC_{50} value of TCS on *O. mossambicus* was calculated based on the 96 h mortality data of fishes exposed to TCS. The data were plotted as graph and there was a positive correlation ($P<0.05$) between the mortality of fishes and concentration of TCS (Fig 1). The calculated 96 h LC_{50} value at 95% confidence limits for TCS for *O. mossambicus* was 740 $\mu g.l^{-1}$. During the exposure period the fishes showed behavioural irregularities such as mucous formation, surfacing, erratic movement, air gulping, hanging at the top and discoloration of skin.

Activity of acetylcholinesterase enzyme

Acetylcholinesterase (AChE) activity is widely being used as a biomarker to study the neurotoxic effects of wide range of toxicants in the aquatic environment. Inhibition of AChE activity results in build up of acetylcholine which causes prolonged excitatory post synaptic potential which negatively impacts the overall behaviour and nervous system of fishes (Banaee *et al.,* 2011). In the present study, the mean AChE activity of brain tissue of tilapia exposed to control medium was 0.345±0.01 $\mu M.min^{-1}.mg.protein^{-1}$. In tilapia, on exposure to 96 h acute toxicity of TCS the AChE activity of the brain got significantly reduced to 0.266±0.03, 0.209±0.02, 0.175±0.01 and 0.088±0.01 $\mu M.min^{-1}.mg.protein^{-1}$ at 131, 262, 523 and 1046 $\mu g.l^{-1}$ respectively (Fig 2). AChE activity in the brain tissue of tilapia was significantly ($p<0.05$) reduced on exposure to different concentrations of TCS. There was no significant difference in enzyme activity between the control and solvent control group of tilapia in the acute toxicity test during the study period. The degree of reduction of enzyme activity was found to be increase with increase in concentration of TCS during the exposure. The reduction of AChE activity in tilapia on exposure to acute concentrations of TCS is shown in Fig 2. Highest amount of inhibition of AChE was observed at 1046 $\mu g.l^{-1}$ of TCS in tilapia. Similar observation of reduction in enzyme activity in brain tissue was observed in zebra fishes by Oliviera *et al.* (2009) and Pullaguri *et al.* (2020).

Activity of glutathione S transferase

Glutathione S transferase is involved in detoxification of xenobiotics at cellular level and their activity in different organs acts as a potential biomarker in toxicity tests (Sturve *et al*, 2008). In the present study, the observed increase in concentration of GST enzyme in liver tissue can be attributed to the detoxification process of toxic metabolites and it is an indicator of defence mechanism (Modesto and Martinez, 2010; Sahu *et al.,* 2018). The mean GST activity in liver tissue

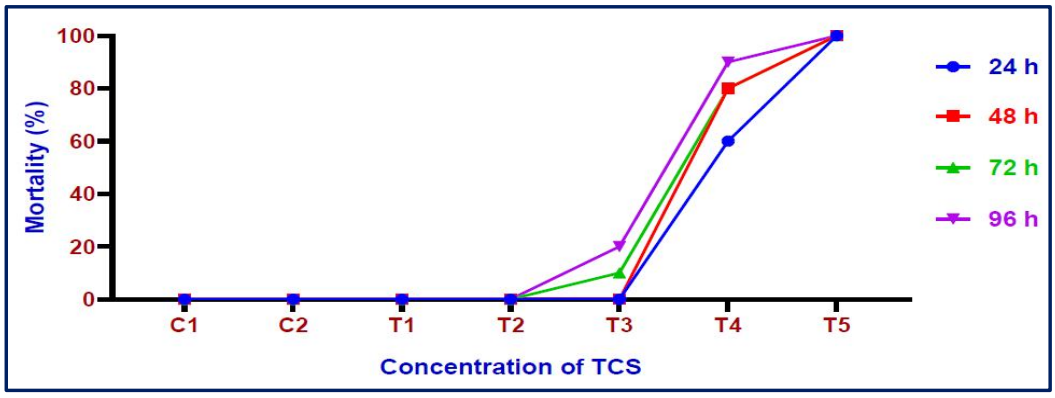

Fig 1: Mortality of *Oreochromis mossambicus* on exposure to different concentration of TCS. C1 – Control; C2 – Solvent Control; T1 – 0.131 ppm; T2 – 0.262 ppm; T3 – 0.523 ppm; T4 – 1.046 ppm; T5 – 2.092 ppm.

of tilapia at control medium during acute toxicity test was 2.637±0.13 n mol. min^{-1} $mg.protein^{-1}$. The GST activity was induced on exposure to TCS in fishes with significant increase in activity with increase in concentration of TCS. The GST activity on exposure to 96 h acute toxicity test significantly increased to 3.022±0.06, 3.346±0.10, 3.860±0.10 and 3.551±0.10 n mol. min^{-1} $mg.protein^{-1}$ at 131, 262, 523 and 1046 $\mu g.l^{-1}$ respectively ($p<0.05$) (Fig 3). Similarly, an elevation in GST activity due to TCS toxicity were reported in zebra fish by Oliviera *et al.* (2009), in sword tail fish by Liang *et al*. (2013) and also in mussels and zooplankton (Canesi *et al.* 2007; Binelli *et al.,* 2011; Peng *et al.,* 2013; Han *et al.,* 2016). GST inhibition at the early larval stages of *Solea senegalensis* observed by Araujo *et al.* (2019) proves that TCS inflicts more damage to fishes at their early life stages.

DNA damage in gill and liver of tilapia exposed to acute and sublethal toxicity of TCS

The integrity of DNA damage in blood cells and different tissues have been proved as potential biomarker for environmental toxicology. In the present investigation,

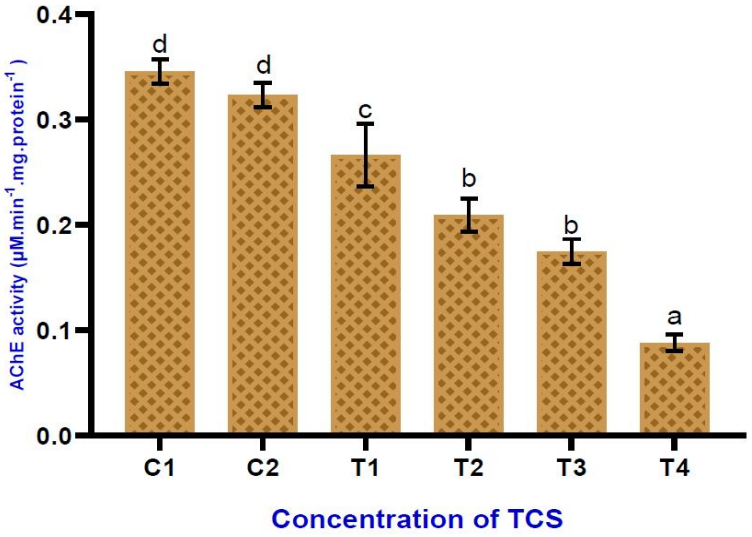

Fig 2: Mean acetylcholinesterase activity in *O. mossambicus* exposed to acute concentration of TCS for 96 h. [Superscripts indicate significant difference ($p<0.05$) between control and different concentrations within the same exposure period. Error bars denote standard error (n=3)].

C1 - Control; C2 - Solvent Control; T1 - 0.131 ppm; T2 - 0.262 ppm;
T3 - 0.523 ppm; T4 - 1.046 ppm.

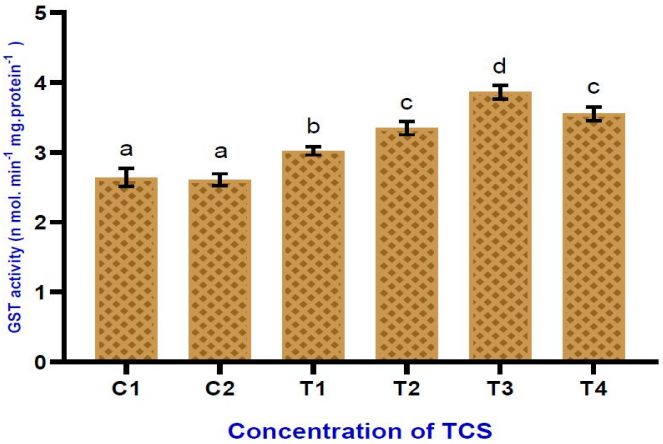

Fig 3: Mean glutathione S transferase activity in *O. mossambicus* exposed to acute concentration of TCS for 96 h. [Superscripts indicate significant difference ($p<0.05$) between control and different concentrations within the same exposure period. Error bars denote standard error (n=3)].

C1 – Control; C2 – Solvent Control; T1 – 0.131 ppm; T2 – 0.262 ppm;
T3 – 0.523 ppm; T4 – 1.046 ppm

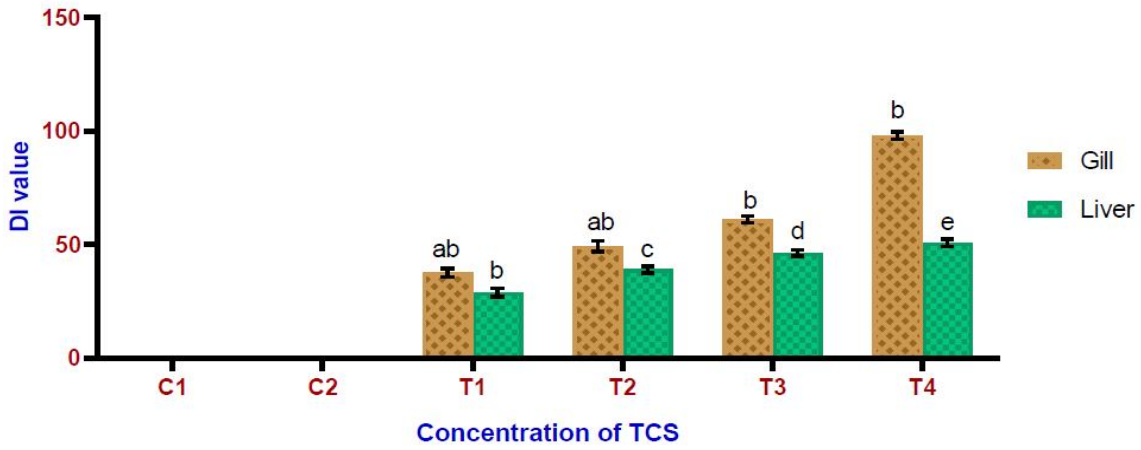

Fig 4: Mean DNA Damage Index (DI) in gill and liver of *O. mossambicus* exposed to acute concentration of TCS for 96 h. [Superscripts indicate significant difference ($p<0.05$) between control and different concentrations within the same exposure period. Error bars denote standard error (n=3)].
C1 – Control; C2 – Solvent Control; T1 – 0.131 ppm; T2 – 0.262 ppm;
T3 – 0.523 ppm; T4 – 1.046 ppm.

significant dose dependent DNA damage intensity was recorded in gill and liver tissues of tilapia and rohu. The difference in extent of DNA damage in various tissues could be attributed to number of alkali – labile sites and variability in DNA of different tissues (Paul *et al.* 2019) because different cells have different levels of DNA single strand breaks due to variation in antioxidant concentration, metabolic and repair activity (Lee and Steinert, 2003). Effect of TCS on the index of DNA damage (DI) on gill and liver tissues of tilapia during acute toxicity test is presented in Fig 4. DNA damage was not observed in the control group of fishes in both gill and liver tissues of tilapia during 96 h acute toxicity test. In gill tissue, the mean DI values were observed as 37.564 ± 1.92, 49.275±2.34, 61.005±1.47 and 98.014±1.69 at 96 h acute toxic concentrations of 131, 262, 523 and 1046 $\mu g.l^{-1}$ respectively. In liver, the mean DI values at the concentrations of 131, 262, 523 and 1046 $\mu g.l^{-1}$ were 28.789±1.86, 38.877±1.39, 46.226±1.32 and 50.705±1.58 respectively. The DNA damage increased with increased in concentration of TCS in gill and liver tissue. The DI value was found to be higher in gill than the liver tissue. The TCS induced genotoxicity study on rainbow trout by Capkin *et al*. (2017) in red blood cells showed significant DNA damage. In addition to fishes, studies by Silva *et al.* (2015) and Xu *et al.* (2015) revealed the TCS induced DNA damage in live food organisms such as *Daphnia magna* and *Artemia salina*. So, the present investigation proved that TCS can affect the target tissue of fishes and induces DNA strand breakage.

CONCLUSION

The toxicity result of the investigation shows that it has the potential to affect the tilapia at the median lethal concentration of 740 $\mu g.l^{-1}$ respectively. It also has the potential to affect the fishes biochemically and genetically. The present detailed investigation on enzyme activity and DNA damage clearly showed that the TCS could alter the normal metabolic processes of the aquatic organisms. So, this preliminary study of TCS determination in fishes would gain interest in regular monitoring to obtain the contamination profile of emerging pollutants of personal care products. So, it is necessary to study in detail about the polluting agents of personal care products, their fate and transportation in the aquatic environment.

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