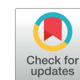
Environmental Pollution 236 (2018) 304–312

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Triclosan affects axon formation in the neural development stages of zebrafish embryos (*Danio rerio*)



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A R T I C L E I N F O

Article history:
Received 19 September 2017
Received in revised form
13 December 2017
Accepted 27 December 2017

Keywords:
Triclosan
Developmental neurotoxicity
Axonogenesis
Zebrafish

A B S T R A C T

Triclosan (TCS) is an organic compound with a wide range of antibiotic activity and has been widely used in items ranging from hygiene products to cosmetics; however, recent studies suggest that it has several adverse effects. In particular, TCS can be passed to both fetus and infants, and while some evidence suggests *in vitro* neurotoxicity, there are currently few studies concerning the mechanisms of TCS-induced developmental neurotoxicity. Therefore, this study aimed to clarify the effect of TCS on neural development using zebrafish models, by analyzing the morphological changes, the alterations observed in fluorescence using HuC-GFP and Olig2-dsRED transgenic zebrafish models, and neurodevelopmental gene expression. TCS exposure decreased the body length, head size, and eye size in a concentration-dependent manner in zebrafish embryos. It increased apoptosis in the central nervous system (CNS) and particularly affected the structure of the CNS, resulting in decreased synaptic density and shortened axon length. In addition, it significantly up-regulated the expression of genes related to axon extension and synapse formation such as *α1-Tubulin* and *Gap43*, while decreasing *Gfap* and *Mbp* related to axon guidance, myelination and maintenance. Collectively, these changes indicate that exposure to TCS during neurodevelopment, especially during axonogenesis, is toxic. This is the first study to demonstrate the toxicity of TCS during neurogenesis, and suggests a possible mechanism underlying the neurotoxic effects of TCS in developing vertebrates.

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1. Introduction

5-Chloro-2-(2, 4-dichlorophenoxy) phenol, known commercially as triclosan (TCS), CH-3565, and Irgasan, is one of the most commonly used antimicrobials; it has a wide spectrum of both antibacterial and antifungal effects. Developed in 1960s, it has been widely used in personal hygiene products such as toothpastes, soaps, and deodorants, as well as in cosmetics, clothes, textiles, and toys (Rodricks et al., 2010). Certain concentrations of TCS have also been reported to be effective against Methicillin-resistant *Staphylococcus aureus* (MRSA), as a result it has been used as an agent for the decolonization of the skin of patients carrying MRSA (Coia et al., 2006). However, recent indications of adverse events have led to the United States' Food and Drug Administration to include it in their list of prohibited antimicrobial ingredients contained in antiseptic washes (Food, 2016).

It has been demonstrated that exposure to TCS can disturb hormone regulation and fat metabolism, and it can induce oxidative stress, apoptosis, inflammation, and carcinogenicity (Ruszkiewicz et al., 2017). It can be transported across the placenta to affect fetal development, and development of infants by accumulating in breast milk (Paul et al., 2012) (Allmyr et al., 2006). Importantly, some reports have been recently proposed that TCS is potentially neurotoxic. For example, prenatal exposure to TCS was found to reduce the circumference of the head among boys at birth (Lassen et al., 2016). The *in vitro* application of TCS to mouse primary cortical neurons results in both aryl hydrocarbon receptor-related apoptosis and the activation of CYP450 enzyme

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https://doi.org/10.1016/j.envpol.2017.12.110
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expression (Szychowski et al., 2016); in addition, the expression of apoptosis-related genes was also increased in rat neural stem cells (Park et al., 2016). This pattern of damage is characteristic of toxins that can impair neural development. However, the effect of TCS on neural development *in vivo* has not yet been fully determined.

The zebrafish (*Danio rerio*) is an *in vivo* experimental animal model that can be used to evaluate developmental neurotoxicity (DNT) (Padilla et al., 2017). The mechanisms underlying zebrafish central nervous system (CNS) development are similar to those of mammals, but zebrafish are smaller and grow much faster. The test substance may be delivered to embryos directly through the environment (i.e. the water they inhabit), so that developmental toxicity can be evaluated without any maternal influences. Moreover, because they are transparent, zebrafish that are transgenic for fluorescent markers can be used to quickly and easily visualize the developmental processes of the CNS, and even allow for the monitoring of neurological defects in live embryos.

Capitalizing on these advantages of the zebrafish model, this study aimed to analyze the potential DNT of TCS *in vivo*; we hypothesized that TCS exposure would cause the zebrafish CNS to appear underdeveloped due to disturbance of a specific neurodevelopmental stage. Therefore, we examined the changes in 1) developmental morphology, 2) CNS apoptosis, 3) CNS neuronal structure, and 4) the expression levels of genes involved in apoptosis and neurogenesis to clarify and better understand the mechanism of DNT induced by TCS exposure to vertebrate embryos.

2. Materials and methods

2.1. Chemical

TCS (Irgasan, CAS 3380-34-5, ≥97.0%), acetone (CAS 67-64-1), and acridine orange (CAS 494-38-2) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). All primers used in this study were synthesized by Cosmogenetech (Daejeon, Korea). Hybrid-R™ (GeneAll, Seoul, Korea) used for RNA isolation and TOPreal™qPCR 2X PreMIX (Enzynomics, Daejeon, Korea) used for real-time PCR were purchased from YMSKorea (Seoul, Korea).

2.2. Zebrafish maintenance and egg acquisition

Zebrafish have been maintained through established methods (Westerfield, 1995). Briefly, the adult zebrafish were raised at a temperature of 28.5 °C ± 1 and 14 h a day, 10 h a night. The pH was 7, and the concentrations of nitrite 0.3 mg/l and nitrate 12.5 mg/l were maintained. Live Brine shrimp were fed into the diet in an amount sufficient to consume within 10 min for three times a day.

Adult zebrafish were used for mating after 3 months of age. A bluish female fish with a plump abdomen and a slim, yellowish male fish were selected. The mating cages were installed at the evening before mating to separate fish from the floor so as not to feed on the eggs that were produced. Female and male fish were divided to 1:1 or 1:2 using dividers. It was removed on the mating day and lights were turned on to induce laying eggs. Spawning eggs were collected using zebrafish embryo medium in about 1 h and used for the experiment according to the established method (Westerfield, 1995).

2.3. TCS exposure

The TCS stock solution was prepared by dissolving TCS in acetone at 6 mg/ml and used for dilution by concentration. It was diluted to 0.2, 0.4, 0.6, and 0.8 mg/l using embryo medium in the survival rate, hatching rate, and heart beats analysis. Control was made by diluting acetone in the same amount at 0.8 mg/l. In the subsequent experiments, TCS was diluted at 0.2, 0.4, and 0.6 mg/l. The same amount of acetone at 0.6 mg/l was dissolved to embryo medium for making control. We also set up negative control using embryo medium without acetone.

Spawning eggs were washed in embryo medium and transferred 10 embryos per well to a 24-well plate (SPLLife Sciences, Seoul, Korea). The embryos were exposed to chemicals with 1 ml of test solution per well. Three independent experiments were performed with 20 embryos per experiment in each group (total 60 embryos per group). The plate was maintained at the same temperature and light conditions as the adult. Dead embryos were identified and removed, and test solutions were changed to fresh solutions once daily for 4 days.

2.4. Analysis of TCS toxic effects on embryo-larvae

All procedures using zebrafish embryos were conducted with the approval of Seoul National University Institutional Animal Care and Use Committee (SNU-171123-1). Survival and hatching rates of embryos and larvae were observed at intervals of 12 hpf up to 96 hpf. The heart beats were measured after 36 hpf and the number of beats per 15 s was counted every 12 hpf. We used an Olympus IX70 optical microscope (Olympus, Japan). We also measured the proportion of morphological abnormalities with the same device at 96 hpf and photographed with a baumer optronic camera (Baumer Ltd., Southington, CT, USA) and ToupView (ToupTek Photonics, Hangzhou, China).

2.5. Morphological analysis of DNT

15 embryos per group were photographed from the side, and the CNS anomalies were evaluated according to the previously reported methods at 96 hpf (Zoupa and Machera, 2017). Briefly, the zebrafish head was divided into three parts: forebrain, midbrain, and hindbrain. The alterations were judged based on the curvature change, increase and decrease in size, and boundary loss of each part. In addition, morphological analysis of neurotoxicity was performed by measuring body length, head size, and eye size. The body length was measured horizontally from the foremost mouth to the tip of the tail. The size of the head was measured to be perpendicular to the long axis from the back of the eye, and the eye size was examined when both eyes were completely overlapped. All measurements were performed using an Olympus IX70 optical microscope, a baumer optic camera and ToupView.

2.6. Analysis of apoptosis in brain and spinal cord using acridine orange

Acridine orange was dissolved in distilled water at a concentration of 5 mg/ml and made into a 1000X stock solution. Zebrafish exposed to concentration-specific TCS for 95 hpf were transferred to an acridine orange solution diluted to 5 μg/ml in embryo medium and incubated for 1 h without light. After 1 h, the acridine orange was removed and the zebrafish washed several times with fresh embryo medium were examined using an Olympus IX70 fluorescence microscope and Nikon D2X (Nikon, Japan). 12 embryos per group were analyzed and the number of cells showing acridine orange positivity in the brain and spinal cord region was compared between groups.

2.7. Nervous system fluorescence analysis in the spinal cord of transgenic zebrafish

The GFP and RFP expression changes of 15 transgenic zebrafish per group were analyzed. At 96 hpf, they were photographed under

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