Interdisciplinary Studies on Environmental Chemistry — Environmental Specimen Bank,
Eds., T. Isobe, K. Nomiyama, A. Subramanian and S. Tanabe, pp. 149–154.

Toxic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) on Peripheral Nervous System of Developing Red Seabream (*Pagrus major*) Embryos

Midori IIDA[1], Eun-Young KIM[2], Yasunori MURAKAMI[3],
Yasuhiro SHIMA[4] and Hisato IWATA[1]

[1]*Center for Marine Environmental Studies (CMES), Ehime University, Matsuyama 790-8577, Japan*
[2]*Department of Life and Nanopharmaceutical Science and Department of Biology, Kyung Hee University, Seoul 130-701, Korea*
[3]*Graduate School of Science and Engineering, Ehime University, Matsuyama 790-8577, Japan*
[4]*National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Imabari 794-2305, Japan*

(Received 13 April 2010; accepted 29 May 2010)

Abstract—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent, bioaccumulative and toxic contaminant widely distributed in the environment. Fishes are the most sensitive vertebrates to TCDD, especially during their early developmental stages. TCDD induces various toxic effects including yolk sac edema, craniofacial malformation and neural damage in developing fish embryos. However, the effects of TCDD on peripheral nervous system (PNS) remain unclear. To clarify how TCDD affects the morphology of PNS in developing red seabream (*Pagrus major*) embryos, the present study investigated the TCDD-induced effects on nervous system using an anti-acetylated tubulin antibody and a HNK-1 antibody. The embryos at 10 h post-fertilization (hpf) were exposed to various concentrations (0–100 μg/L) of TCDD in seawater for 80 min. The PNS of craniofacial region was completely disrupted at 120 hpf in 12.5 μg/L TCDD-treated embryos, whereas no significant effect in trunk PNS was observed. As for neural crest cells (NCC) and their differentiated tissues, the hindbrain segments could not be clearly seen at 48 hpf in TCDD-exposed embryos. These results suggest that TCDD may have a specific effect on developing craniofacial PNS that might be initiated by disrupting NCC differentiation and/or its migration.

Keywords: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, peripheral nervous system, red seabream

INTRODUCTION

Planar halogenated aromatic hydrocarbons (PHAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and polychlorinated

biphenyls (PCBs) are highly lipophilic and persistent environmental pollutants that undergo bioaccumulation in the food chain.

It has been documented that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), one of the most potent PHAHs, elicits reproductive and developmental toxicity, immunotoxicity, cardiotoxicity, neurotoxicity, hepatotoxicity, wasting syndrome, and lethality in vertebrates (Peterson *et al.*, 1993). These toxic effects exerted by PHAHs are of great concern in a variety of wildlife species. The toxicity is mainly mediated by a transcriptional factor, the aryl hydrocarbon receptor (AHR), which regulates the multiple target genes including cytochrome P4501A (CYP1A) (reviewed by Mimura and Fujii-Kuriyama, 2003). Bony fish is one of the most sensitive vertebrates to TCDD and exhibits developmental defects such as mortality, yolk sac edema, pericardial edema, and craniofacial malformation (Guiney *et al.*, 1997; Teraoka *et al.*, 2002, 2006; Hill *et al.*, 2004). Most studies on TCDD-induced toxicity were conducted in fresh water and euryhaline fishes. In contrast, there is little information on TCDD toxicity in marine fishes.

To investigate TCDD toxicity in marine fishes, we have focused on seabream (*Pagrus major*) (Yamauchi *et al.*, 2006). The red seabream is one of the most popular commercial species in Japan, belongs to the order of Perciformes (the family sparidae) and inhabits Asian coastal and continental shelf areas. Due to their coastal habitation, higher trophic position in the food web and long-life span (15–20 years), high risk of the exposure to PHAHs in red seabreams is of concern. Furthermore, our previous study showed that 50% lethal concentration (LC_{50}) value for the red seabream was 0.36 pg/g at 96 hpf. This indicates that the red seabream is one of the most sensitive fishes to TCDD toxicity (Yamauchi *et al.*, 2006).

Multiple reports have demonstrated that TCDD exposure induces apoptosis and reduction of the capacity and the number of axon tracts for the brain development in fish embryos (Dong *et al.*, 2001, 2002, 2004; Hill *et al.*, 2003; Ton *et al.*, 2006). It is also known that toxicant-induced impairment of neurosensory functions affects behavioral traits of exposed organisms at sublethal toxic concentrations (Froehlicher *et al.*, 2009). However, the effect of TCDD exposure on peripheral nervous system (PNS) is not well understood. PNS is a part of the nervous system consisting of nerves and ganglia outside the brain and the spinal cord. PNS neurons and glia differs from neural crest cells (NCC) that preferentially migrate into the anterior sclerotome halves (Schwarz *et al.*, 2009).

In the present study, we investigate the effects of TCDD exposure on peripheral nervous system in marine fish. Red seabream embryos were treated with graded concentrations of TCDD and the development of PNS was monitored with an immunostaining method.

MATERIALS AND METHODS

Chemicals

2,3,7,8-Tetrachrolodibenzo-*p*-dioxin was obtained from Wellington Laboratories Inc.

Red seabream eggs

Fertilized eggs were obtained from naturally reproducing red seabreams aqua-cultured at Hakatajima Station, National Center for Stock Enhancement, Fisheries Agency in Ehime Prefecture, Japan. Eggs were collected within 6 hrs after spawning, placed in an aerated seawater tank, and transported to the laboratory. The normally developing embryos of red seabreams floated on the water surface, while the dead or unfertilized eggs deposited on the bottom of tank (Sakai *et al*., 1985). Following slow stirring of water with a glass rod for a few minutes, floating eggs were collected and used for further experiments. Pigmentation was prevented *in vivo* by treatment with 0.003% phenylthiourea.

Waterborne exposure to TCDD

Eggs were maintained at 19.5 ± 1.5°C in TCDD-free water. The water was stirred slowly with a glass rod every hour in order to avoid bacterial multiplication on eggs. Conditions of TCDD treatment including concentrations and exposure periods were as followed in the study of Yamauchi *et al*. (2006). The freshly fertilized eggs (4 g of eggs per dose) at 10 hpf (hours post fertilization) were exposed to 10 ml of seawater containing no vehicle, vehicle (0.008% toluene) or graded concentrations of TCDD (3.1, 6.2, 12.5, 25, 50 or 100 μg/L). After 80 min exposure, eggs were removed from the TCDD solutions, rinsed in TCDD-free seawater, and transferred into 1 L beaker containing 800 ml of TCDD-free seawater. After the hatching of eggs, the seawater was changed every day.

Immunohistochemistry

To visualize the PNS and NCC in red seabream embryos, immunohistochemistry was carried out. All the procedures were done at room temperature. After the discoloration of embryos, they were washed three times with Tris-buffered Saline with 0.1% Tween20 (TBST)/5% DMSO for 30 min. Samples were then subjected to overnight blocking with 5% skim milk in TBST/DMSO (TSTM), and incubated for 4 days with 1000 × diluted primary antibody (anti-acetylated tubulin (SIGMA) antibody for PNS or HNK-1 antibody (BECTON DICKINSON) for NCC). The samples were washed 6 times with TBST/DMSO for 1 hr and incubated for 2 days with a 200 × diluted secondary antibody (goat anti-mouse IgG for anti-acetylated tubulin antibody or goat anti-mouse IgM (zymed) antibody for HNK-1 antibody. Following the incubation, the embryos were washed 12 times with TBST for 30 minutes for each washing. The PNS and NCC were visualized by incubating the embryos with 3′,3′-diaminobenzidine (DAB) in TBST containing 0.01% H_2O_2, after pre-incubation with DAB in TBST for 30 min.

RESULTS AND DISCUSSION

To understand the effects of TCDD exposure on the PNS and NCC in the early life stage of red seabream embryos, the nerve cells were visualized using anti-acetylated tubulin antibody and HNK-1 antibody, respectively.

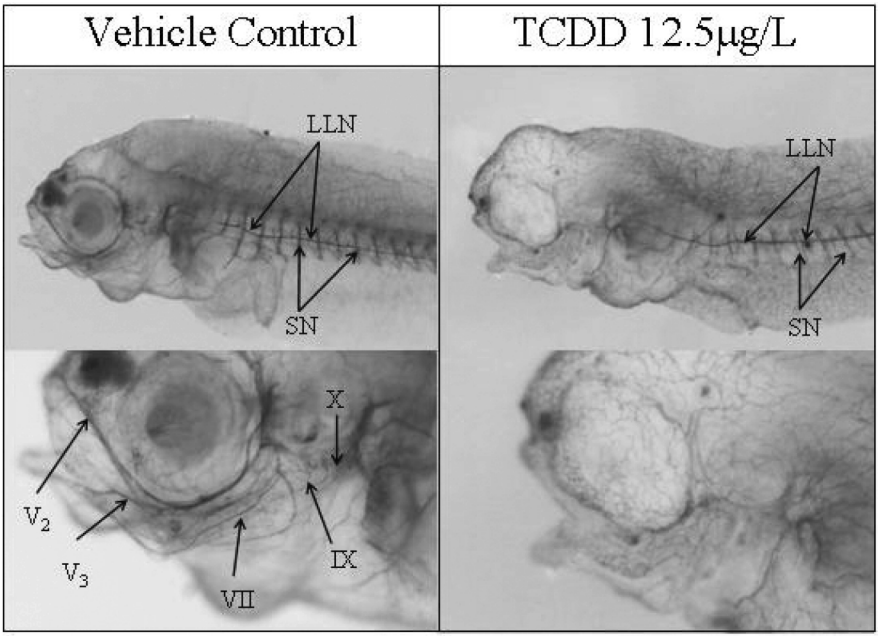

Fig. 1. Immunohistochemistry using an anti-acetylated tubulin antibody for 136 hpf-embryos. Left: vehicle-treated embryo. Right: 12.5 μg/L TCDD-treated embryo. LLN: lateral line nerve. SN: spinal nerve. V2: maxillar nerve. V3: mandibular nerve. VII: facial nerve. IX: glossopharyngeal nerve. X: vagus nerve. While LLN and SN were normally developed in vehicle control and TCDD-treated embryos, the formation of cephalic PNS was distrupted in TCDD-treated embryos.

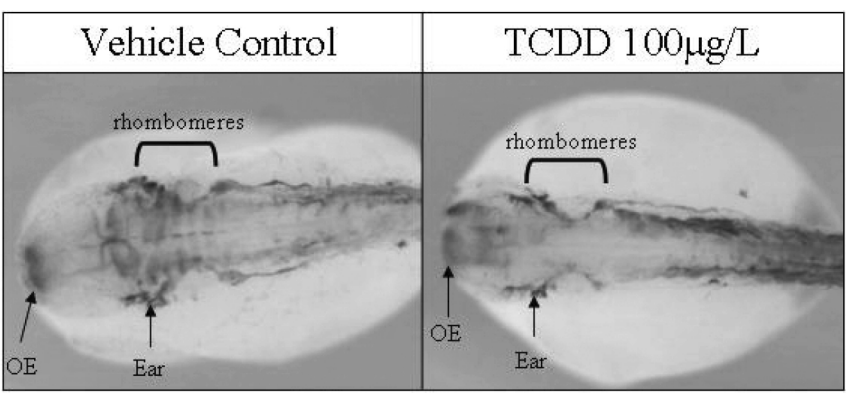

Fig. 2. Immunohistochemistry using a HNK-1 antibody for 48 hpf embryos. Left: vehicle-treated embryo. Right: 100 μg/L TCDD-treated embryo. OE: olfactory epithelium. While apparent structure of rhombomeres were observed in vehicle control embryos, no rhombomeres were found in TCDD-exposed embryos.

At 39 hpf spinal nerve (SN), posterior lateral line nerve (PLLN), medial longitudinal fasciculus and the tract of the post optic commissure (TPOC) are visible. No defects in those nerves were observed in spite of the TCDD-treated embryos (data not shown).

The maxillary nerve (V_2), the mandibular nerve (V_3), the facial nerve (VII), and the glossopharyngeal nerve (IX) appear in vehicle embryo at 78 hpf. In TCDD exposed embryos, the nerve located in branchial region showed suppressed nerve development or could not be seen clearly.

We observed the normal growth of trigeminal nerve (V), VII, IX and vagus nerve (X) in solvent control embryos at 136 hpf. All the major branches have appeared by this stage. In contrast to these embryos, the growth of cranial PNS was disturbed in TCDD-treated embryos, whereas the trunk PNS appeared to be less affected by TCDD (Fig. 1). These results indicated that TCDD exposure affects nerve development in the branchial region from 78 hpf.

In fish, TCDD exposure exerts craniofacial defects. Teraoka *et al*. (2006) demonstrated that TCDD may impair jaw growth through down regulation of sonic hedgehog (Shh). In addition Shh inhibit the apoptosis of NCC in branchial region (Brito *et al*., 2006).

Immunostaining using the HNK-1 antibody indicated that most TCDD-treated embryos lost the nerve root derived from NCC (Fig. 2). Hence, this suggests that TCDD inhibits the differentiation and proliferation of NCC. Thus, the effect of TCDD on NCC may lead to poor formation of PNS in the anterior region of embryos. On the other hand, Grimes *et al*. (2008) demonstrated that neural crest-derived tissues are affected by PCB126, one of PHAH congeners, while this phenotypic alteration does not arise from an impairment of neural crest migration, patterning or differentiation. These results suggest that NCC can migrate to the correct positions, but fail to grow.

The present study showed the effects of TCDD exposure on the PNS in developing red seabream embryos. However, the fact that cephalic PNS is more disrupted than the trunk PNS remains unclear. Further studies are necessary to clarify the mechanisms of TCDD-induced neurotoxicity.

Acknowledgments—The authors thank Prof. An. Subramanian, Ehime University, for critical reading of this manuscript. This study was supported by Grant-in-Aid for Scientific Research (S) (21221004) to Hisato Iwata from Japan Society for the Promotion of Science. Financial assistance was also provided by "21st Century Global COE Program" from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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M. Iida (e-mail: red_green@agr.ehime-u.ac.jp)