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

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Abstract—Bony fish is highly sensitive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure, especially at 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 the peripheral nervous system (PNS) of fish remain unclear. To clarify whether TCDD affects the morphological abnormality of PNS, the present study investigated the development of PNS in TCDD-treated red seabream (*Pagrus major*) embryos using a fluorescein isothiocyanate (FITC) anti-acetylated tubulin antibody. The embryos at 10 hrs post-fertilization (hpf) were exposed to 0, 0.1, 0.4 or 1.7 μg/L of TCDD in seawater for 80 min. The PNS in each exposed group was observed at 48, 78, 120 and 136 hpf. The axon guidance of posterior lateral line nerve (PLLN) was less affected by 0.1 or 0.4 μg/L of TCDD exposure, but slightly damaged by 1.7 μg/L of TCDD. On the other hand, the craniofacial distribution of PNS was notably disrupted in 1.7 μg/L TCDD-treated embryos at 120 and 136 hpf. The glossopharyngeal nerve (IX) and vagus nerve (X) were abolished by TCDD in a dose-dependent manner. At 120 hpf and 136 hpf, 1.7 μg/L TCDD exposure also affected the spinal nerve (SN), and inhibited the formation of nerve fascicle. These observations showed that TCDD produces specific effects on the development of craniofacial PNS in red seabream embryos even at low concentration (0.1 μg/L).

Keywords: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), peripheral nervous system (PNS), developmental toxicity, red seabream

INTRODUCTION

Dioxins and related compounds (DRCs) including 2,3,7,8-tetrachlorodibenzo-*p*-

dioxin (TCDD) elicit a wide range of toxic responses in a variety of vertebrate species. Toxicity of dioxin is mainly mediated by a transcriptional factor, aryl hydrocarbon receptor (AHR), which regulates the transcription of multiple target genes including cytochrome P4501A (CYP1A). Bony fish is one of the most sensitive vertebrates to TCDD exposure and exhibits developmental defects such as edema and malformation (Carney *et al*., 2006; Teraoka *et al*., 2006; Mehta *et al*., 2008). TCDD-induced toxicities have been investigated at early stages of various fish species (Henry *et al*., 1997; Hornung *et al*., 1999; Toomey *et al*., 2001). Several studies have reported that the development of central nervous system (CNS) in vertebrates is affected by TCDD (Dong *et al*., 2002; Ton *et al*., 2006). However, no investigation of the effects of TCDD exposure on peripheral nervous system (PNS) has been reported. PNS is a part of the nervous system consisting of nerves and ganglia other than the brain and spinal cord. Chemical-induced impairment of neurosensory functions may affect behavioral traits of exposed organisms (Froehlichera *et al*., 2009).

The red seabream (*Pagrus major*) belongs to the order of Perciformes (Family Sparidae) and is one of the most popular commercial fish in Japan. The high risk of DRCs to this species is a matter of concern due to their habitat in coastal areas, the higher trophic level and their long-life span.

In this study, we investigate the effects of TCDD exposure on the PNS in the red seabream. The embryos were treated with graded concentrations of TCDD and the development of the PNS was monitored by immunostaining.

MATERIALS AND METHODS

Chemical

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (50 μg/mL in nonane) was obtained from Wellington Laboratories Inc.

Red seabream eggs

Fertilized eggs of the red seabream were obtained from a naturally reproducing aqua culture system at Hakatajima Station, National Center for Stock Enhancement, Fisheries Agency in Ehime Prefecture, Japan. Eggs were collected within 6 hours after spawning, placed in aerated seawater tank, and transported to the laboratory. According to Sakai *et al*. (1985), following the slow stirring of water with a glass rod for a few minutes, floating eggs were collected as normally developing embryos and used for further experiments (deposited eggs were considered dead or unfertilized eggs).

The pigmentation of embryos was blocked *in vivo* by treatment with 0.003% phenylthiourea.

Waterborne exposure to TCDD

Red seabream embryos were maintained at 19.5 ± 1.5°C in TCDD-free seawater. The seawater was stirred slowly with a glass rod every hour in order to

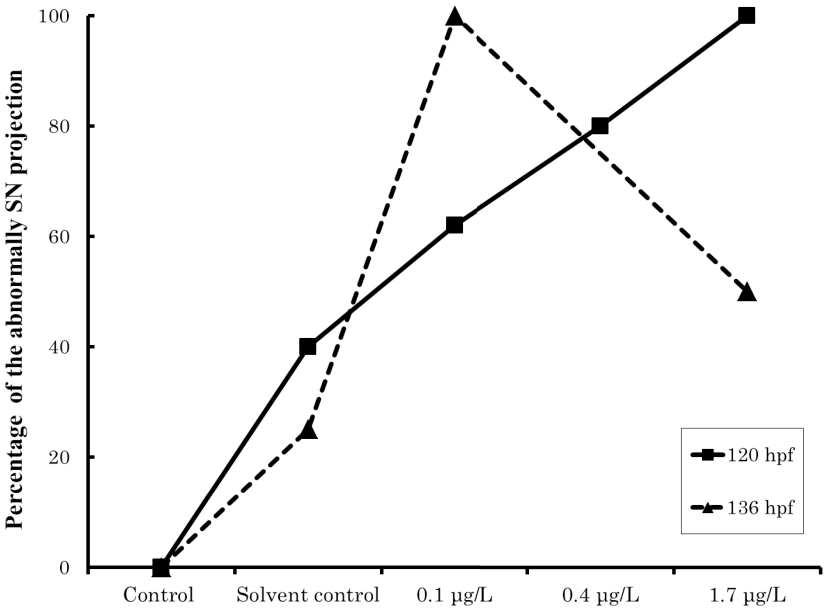

Fig. 1. Effects of TCDD on the SN projection in TCDD-exposed red seabream embryos. Two to ten embryos were subjected to the observation in each group.

prevent eggs from the bacterial multiplication. After the hatching, the seawater was changed every day. TCDD treatment was conducted following the method of Yamauchi *et al*. (2006). The freshly fertilized eggs at 10 hpf (hours post fertilization) (4 g per dose) were exposed to 1 ml seawater containing no vehicle (control), vehicle (toluene as solvent control) or graded concentrations of TCDD (0.1, 0.4 and 1.7 μg/L). After 80 min exposure, embryos were removed from the TCDD solutions, rinsed in TCDD-free seawater, and transferred into 1 L beaker containing 800 ml TCDD-free seawater.

Immunohistochemistry

To visualize the PNS of hatched red seabreams, immunohistochemistry was carried out using an anti-acetylated tubulin antibody. After discolorations, embryos were washed three times with Tris-buffered saline (TBS)/dimethyl sulfoxide (DMSO) for 30 min at room temperature. Samples were then subjected to overnight blocking with 5% skim milk in TBS/DMSO and then incubated for 4 days with a primary antibody (1000 × diluted anti-acetylated tubulin antibody (Sigma, T7451)) at room temperature. The samples were washed 6 times with TBS/DMSO for 1 hour and incubated for 2 days with a secondary antibody (200 × diluted fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG

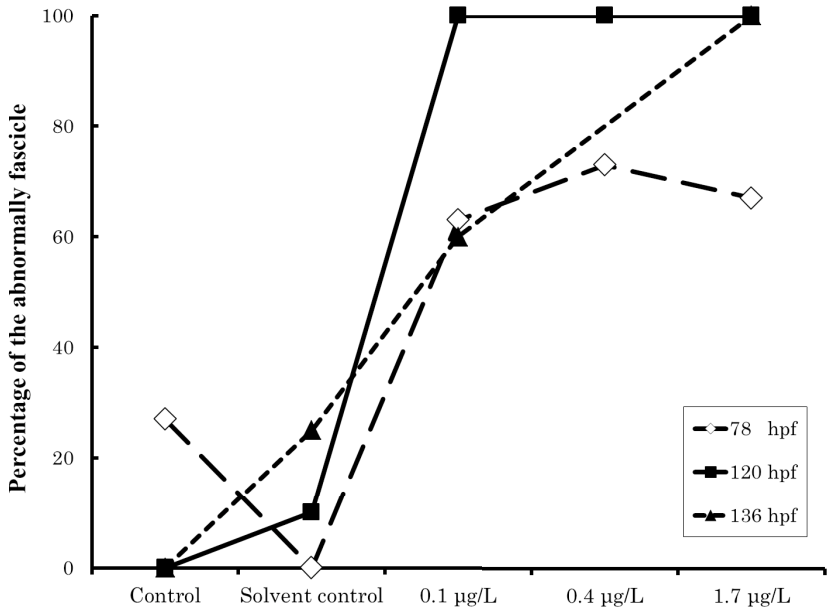

Fig. 2. Effects of TCDD on the nerve fascicle in TCDD-exposed red seabream embryos. Two to ten embryos were subjected to the observation in each group.

(ZYMED Lab. Inc., 62-6511)) at room temperature. Following the incubation, embryos were washed 12 times with TBS/DMSO for 30 min at room temperature. To obtain the digital image, a confocal laser scanning microscope (LSM510, Carl ZEISS) was used.

RESULT AND DISCUSSION

The posterior lateral line nerve (PLLN) of fish is on the body and tail along the anteroposterior axis. Therefore we detect the nerve deviated from body axis as abnormal. Treatment with 0.1 μg/L or 0.4 μg/L of TCDD showed no remarkable effect on the PLLN in all the observed stages of embryos, but 1.7 μg/L of TCDD gave slight damage to the PLLN of embryos. The percentage of abnormality was 0% in control, 20% in solvent control, 0% in 0.1 μg/L, 0% in 0.4 μg/L and 50% in 1.7 μg/L at 120 hpf.

The spinal nerve (SN) branched off from the spinal cord and is located between somites. In control and solvent control embryos, the SN normally projected at 120 hpf and 136 hpf. On the other hand, the SN projected out of somites in TCDD-exposed embryos (0.1, 0.4 and 1.7 μg/L). The number of defected SN increased in a TCDD dose-dependent manner (Fig. 1). The nerve fascicle was clearly observed in control and solvent control embryos. However,

the nerve fascicle formation extended discretely in 0.1, 0.4 and 1.7 μg/L of TCDD-exposed embryos at 78, 120 and 136 hpf (Fig. 2).

The normal development of trigeminal nerve (V) with three major branches, ophthalmic nerve (V_1), maxillary nerve (V_2) and mandibular nerve (V_3), was observed in control, solvent control and 0.1 μg/L TCDD-treated embryos at 120 hpf and 136 hpf. In 0.4 and 1.7 μg/L TCDD-treated exposed embryos, the V was not observed clearly. Similarly, the glossopharyngeal nerve (IX) and vagus nerve (X) were disrupted in TCDD-treated embryos at 120 hpf and 136 hpf in a dose-dependent manner; the percentage of abnormality was 11% in control, 17% in solvent control, 33% in 0.1 μg/L and 70% in 1.7 g/L at 120 hpf. The length of V_1, V_2, V_3, VII, IX and X nerves were measured, but there was no significant change in the length by TCDD exposure.

The present study showed the effects of TCDD exposure on the PNS in developing red seabream embryos. TCDD treatment disrupted the projection of SN, V, IX, and X as well as the formation of nerve fascicle. Intriguingly, the SN disruption was observed in 0.1 μg/L TCDD-treated embryos. This concentration is lower than the TCDD concentration which causes the morphological abnormalities, suggesting that the PNS of red seabream embryos is highly sensitive to TCDD.

Given that TCDD disrupted the nerve guidance, the neural guidance proteins like semaphorin and neuropilin, are considered to be targets of TCDD. We still do not know whether TCDD affects the PNS directly or not. Several previous studies have reported the disruption of connective tissues by TCDD (Kawamura and Yamashita, 2002; Teraoka *et al*., 2010). Since it is known that the neural guidance proteins in the connective tissue play an important role in the signal transmission (Pasterkamp and Giger, 2009), damages in connective tissues might be caused by the altered expression of these proteins and consequently lead to the modulation of signal transmission.

CONCLUSIONS

The axon of PLLN appeared to be slightly changed even in 1.7 μg/L TCDD-treated red seabream embryos. TCDD affected the projections of SN, V, IX and X in a TCDD dose-dependent manner. The formation of nerve fascicle was interfered by TCDD at 120 and 136 hpf.

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