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Developmental toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in artificially fertilized crucian carp (*Carassius auratus*) embryo

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HIGHLIGHTS

- Artificially fertilized crucian carp embryos were successfully fertilized.
- Developmental toxicity in a crucian carp model was characterized for TCDD.
- Different signs of TCDD toxicity were identified during the early life stage.

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent bioaccumulative environmental contaminant that is an endocrine disruptor. Embryos of various fish species are responsive to TCDD and have been used as an alternative method to the acute toxicity test with juvenile and adult fish. The TCDD test has similar endpoints of developmental toxicity. However, their sensitivity and signs of TCDD-induced toxicity are different depending on fish species and its habit. Crucian carp (*Carassius auratus*) – the sentinel species for persistent organic pollutants and a common foodfish in China, Japan, and Korea – was used to identify the developmental toxicity of TCDD. We obtained the fertilized eggs from the artificial fertilization of crucian carp (97.45% success rate). Embryos at 3 h post fertilization (hpf) were exposed to no vehicle, vehicle (dimethylsulfoxide, 0.1% v/v) or TCDD (0.128, 0.32, 0.8, 2 and 5 μg/L) for 1 h and then fresh water was changed and aerated. Embryonic development and toxicity were monitored until 150 hpf. TCDD-exposed group showed no effects on embryo mortality and hatching rate from 6 to 126 hpf. On the other hand, the post-hatching mortality rate in TCDD-exposed group was increased in a dose-dependent manner, especially at high doses (0.8, 2 and 5 μg/L). The LD50 for larval mortality was calculated to 0.24 ng TCDD/g embryo. Pericardial edema was continuously observed in larvae of TCDD-exposed groups from hatching complete time (78 hpf), followed by the onset of yolk sac edema. Hemorrhage and edema showed a significant increase depending on exposure concentration and time. Expression of TCDD-related *CYP1A* genes was evaluated quantitatively. Embryo and larvae in TCDD-exposed groups displayed a significant increase of *CYP1A* gene expression. Overall, we defined TCDD-induced toxicity in artificially fertilized crucian carp embryo and these results suggest that crucian carp can be applied as an early life stage model of TCDD-induced toxicity.

1. Introduction

Dioxins that include polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs), are persistent, lipophilic environmental contaminants that bioaccumulate in fish, causing toxicity (Cook et al., 1990; Tanguay et al., 2003; Walker and Peterson, 1994). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent type and is a known endocrine disruptor in the environment. TCDD is insoluble in the water, however, it is also widespread in water environment due to its high persistency. It may also be produced in thermal processes such as incineration. In fish, TCDD has a high bioconcentration factor and also can be bio-accumulated through the food chain, therefore dioxin levels in fish are much higher than that of the surrounding environment. Bioaccumulation factors (BAF) of TCDD in surface waters and freshwater fish are reported as 11,500 to 24,600 for smallmouth fillets, from 17,900 to 28,300 for brown trout fillets, from 3,000 to 7,500 for white perch fillets, and from 78,500 to 106,000 for white sucker whole bodies which are high enough to concern (Frakes et al., 1993). These values are based on

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concentrations of TCDD in various environment such as water, sediment, and food.

The potential for dioxins to cause reproductive and developmental toxicity using fish embryos has been recognized and studied with various freshwater fishes for many years. Fish embryos are the most sensitive vertebrates to the toxic effects of TCDD and exhibit similar signs of toxicity as other vertebrates (Peterson et al., 1993). The fertilized embryos of fish exposed to TCDD cause cardiovascular dysfunction, edema, hemorrhage, craniofacial malformation, growth arrest and mortality in larvae (Heideman et al., 2005; Henry et al., 1997; Hill et al., 2005; Peterson et al., 1993; Spitsbergen and Atchison, 1991; Spitsbergen et al., 1988; Walker et al., 1991).

The fish embryo toxicity (FET) test is an OECD test guideline and is intended to define the toxicity of chemicals on embryonic stages of fish as an alternative test method to the acute toxicity tests with juvenile and adult fish. The external development of oviparous fish allows their developmental processes to be followed in the absence of maternal influences (Hahn, 2001). Since many fish species are small and have short reproductive cycles, they are relatively inexpensive to maintain and are suitable for multigenerational studies (Hahn, 2001). In addition, observation of development and morphological toxicity is facilitated by the transparent nature of many fish embryos. Due to such characteristics, fish are emerging as important organisms in reproductive and developmental toxicology and in studies of hormonally active agents (endocrine disrupters) (Bonaventura, 1999; Rolland, 1997). Native fish is important to assess contamination of the local aquatic environment. Crucian carp (*Carassius auratus*) is an indigenous species in Korea which is the nationwide fish and commonly used as a sentinel species for the biomonitoring of endocrine disruptor in freshwater (Oh et al., 2009a). We have previously studied the cloning and characterization of dioxin-related genes, such as *CYP1A*, in crucian carp (Oh et al., 2009a,b; Park et al., 2010). However, little is known about the developmental toxicity stimulated by TCDD and species sensitivity to TCDD.

To identify the developmental toxicity of TCDD in crucian carp, we evaluated developmental toxicity, hatching, mortality, morphological toxicity and TCDD-related *CYP1A* gene expression during development in fertilized eggs exposed to TCDD.

2. Materials and methods

2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (99% pure; 10 μg/mL in Toluene) was obtained from Supelco (Bellefonte, PA, USA). TCDD was evaporated under a steam of gaseous nitrogen and dissolved in 100% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 0.128, 0.32, 0.8, 2.5, and 5 mg/L. [^{3}H] TCDD (specific activity, 27.5 Ci/mmol, >97% radiochemical purity by high-performance liquid chromatography) was purchased from EaglePicher Pharmaceutical (Lenexa, KS, USA). All other chemicals were reagent grade and obtained from commercial sources.

2.2. Artificial fertilization of crucian carp and incubation of embryos

Crucian carp obtained from the Inland Fisheries Special Research Institute (Cheongpyeong, Korea) was raised and maintained in dechlorinated water (pH 6.8–7.5, 20 ± 1 °C) with 50% water changes once a week or as necessary to prevent a build-up of harmful nitrate. All the experimental protocols were followed by Korean Animal Protection Law by Ministry of Food, Agriculture, Forestry and Fisheries, Korea.

Through artificial fertilization, eggs were collected. Eggs from crucian carp females injected 2000 IU/kg chorionic gonadotropin (DAESUNG Bio, Korea) and male sperm were mixed in a stainless steel mixing bowl in the absence of water to improve the fertilization ability and avoid attachment to the experimental bowl. Sticky eggs (100–300) were spread on a glass jar. Fertilized eggs were maintained in our laboratory at 23 ± 1 °C in egg water under a 16 h light/8 h dark photoperiod.

2.3. Waterborne exposure of embryos to TCDD

Newly fertilized eggs were selected prior to TCDD exposure. Fertilized embryos at approximately 3 h post fertilization (hpf) were exposed to 600 mL of water containing no vehicle, vehicle (0.1% DMSO) or TCDD (0.128, 0.32, 0.8, 2 and 5 μg/L) previously dissolved in DMSO, with each treatment conducted in triplicate. All treatment contained equal volume of DMSO which is 0.1%. Following the 1 h static TCDD exposure, the embryos from each jar were rinsed three times with TCDD-free water. Embryonic development and toxicity were monitored at 3, 6, 12, 18, 24, 30, 42, 54, 78, 102, 126, and 150 hpf. During the experiment periods, sufficient aeration of the water was maintained. The water was replaced once per day for the duration of the study. The embryos were monitored to assess the developmental stage, heartbeat, hatchability, signs of developmental toxicity and mortality. The embryos were fixed at 4 °C in 2.5% glutaraldehyde as times shown in Fig. 2. To quantify TCDD-induced genes, 10 randomly selected embryos or larvae were frozen in liquid nitrogen and stored at −80 °C until total RNA isolation.

2.4. Quantification of TCDD uptake in embryos

Crucian carp embryos were exposed to radiolabeled [^{3}H] TCDD (27.5 Ci/mmol) to evaluate cellular uptake of TCDD (50 embryos/10 mL water, embryo wet weight: 4.17 mg). Fifty embryos were exposed to 10 mL TCDD (0.8, 2, and 5 μg/L) for 1 h in 100 mL glass beakers at 20 ± 1 °C. After exposure, embryos were washed two times with dechlorinated water. TCDD uptake was measured using a LS-6500 liquid scintillation counter (Beckman Coulter, Palo Alto, CA, USA) with liquid scintillation cocktail (PerkinElmer, Waltham, MA, USA). The relationship between the TCDD water concentration (x = μg/L) and TCDD tissue doses (y = ng TCDD/g embryo, wet weight) was calculated.

2.5. Signs of TCDD-induced developmental toxicity

Embryos or larvae were observed each from 6 hpf to 150 hpf through an Olympus SZ61TRC microscope. Micrographs were taken at 30× with the embryos and larvae in a lateral orientation at the same resolution. In this study, all results were not statistically different between no vehicle and vehicle group. Therefore, vehicle (0.1% DMSO) exposed group was considered as control group.

2.5.1. Developmental stage and heartbeat

The developmental stage was determined by checking for the condition of each embryo at each time point (Fig. 1). The heartbeat rate was calculated by direct observation for 30 s. Three embryos were randomly chosen at each group from 78, 102 and 126 hpf, when the embryos already had distinct heartbeats.

2.5.2. Hatchability and mortality

The hatching time course was determined by counting the number of viable larvae hatched from 54 hpf, when the first embryos hatched, to 78 hpf, when 100% of all groups had hatched. The hatching rate was calculated as the percentage of hatched larvae for each fertilized embryos. Dead and viable embryos were recorded in each group during the experiment period. Embryonic death was determined by ceased development, which was evident as embryos white in color and detached from the glass jars (OECD TG 210 and 212). The dead embryos were removed from jars as soon as death was detected. The post-hatching mortality at 150 hpf was expressed as the percentage of the accumulated dead embryos or larvae at each time to all hatched larvae.