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Developmental Toxicity and EROD Induction in the Japanese Medaka *(Oryzias latipes)* Treated with Dioxin Congeners

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Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are widespread environmental contaminants. These two classes of compounds have been identified as persistent environmental pollutants due to their high hydrophobicity. With eight-substitution positions to react with chlorine atoms, PCDDs and PCDFs have 75 and 135 congeners, respectively. Both the physical and biological properties differ for each congener. The most extensively studied congener of all PCDDs and PCDFs isomers is 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), which is also the most potent (McConnell et *al*. 1978). The potency of other congeners has been graded into toxic equivalent factors (TEFs) based on their relative toxicity to TCDD, which was designated as 1 (Safe 1990).

Although different agencies use different TEFs for different isomers to determine the TCDD toxic equivalent (TE) of a dioxin mixture, those TEFs are usually based on either the acute or subchronic toxicological data from mammals, or *in vitro* enzyme (cytochrome P-4501A) induction. However, aquatic animals may have different TEFs due to the species difference (Walker *et al*. 1991). For quantitative risk assessment other than human, this variation could result in either over- or underestimation of the risk associated with the exposure to the dioxin mixture.

Fish are also reported to be very sensitive to TCDD, especially the developing embryos and larvae (Wisk and Cooper 1990, Walker *et al*. 1992). The Japanese medaka's *(0ryzias latipes)* early life stage (ELS) assay has been used to assess the developmental toxicity of different chemicals (including TCDD) and environmental samples (Wisk and Cooper 1990, Cooper *et al*. 1993). Medaka embryos exhibit basal level of cytochrome-P4501A activity as measured by the aryl hydrocarbon hydroxylase (AHH) reaction, and can be induced by TCDD (Wisk and Cooper 1992). In this study, the potency of other 2,3,7,8-substituted dioxin congeners were determined, as well as the TEFs for medaka, based on the dioxin-induced developmental effects in embryos and the inducibility of 7-ethoxyresorufin *O*-deethylation (EROD) in larvae.

MATERIALS AND METHODS

Tritium-labelled 2,3,7,8-TCDD (20.5 Ci/mmole) was purchased from Chemsyn

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Science Laboratories (Lenexa, KS). 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HeCDD), and 2,3,7,8-tetrachloro-dibenzofran (TCDF) were all purchased from Cambridge Isotope Laboratories (Woburn, MA). Stock solution of each congener was prepared by dissolving the crystalline powder into acetone except TCDD, which was originally dissolved in DMSO and with the further dilutions in acetone. The TCDD concentrations in solution were determined by scintillation counting, but expressed in nominal concentrations in calculation or statistical analysis. Concentrations of other congeners were nominal concentrations.

Twenty to thirty medaka embryos were exposed to graded concentrations of different congeners. Each egg was placed into a Teflon® capped vial containing 1 ml of rearing solution into which the congeners was dissolved. Control animals were in either rearing solution or 0.1% of acetone/rearing solution. Exposure of the embryos began on day zero of development, within 1 to 2 hours after fertilization. The embryos were stored in an incubator at 25°C. The embryos were examined daily under a dissecting microscope for the appearance of any cardiovascular lesion, severe lesions resulting in the death of animals prior or at hatching, hatching success, and 3-day post-hatch mortality.

A group of fifty to sixty larvae was incubated in a rearing dish containing 100m1 solution of different concentrations of dioxin congeners for 72 hours at room temperature. The control group was exposed to rearing solution with less than 0.01% of acetone. At day 3, larvae were sacrificed for the microsomal fraction for the EROD assay (Prince 1993). Protein contents were determined by the BioRad® protein assays (Bradford 1976). At least two tests were performed for each congener.

The LC_{50}s and 50% effective concentrations (EC_{50}s) with 95% confidence interval were calculated using a modified EPA's Probit Analysis Program. Analysis of covariance (ANCOVA) was used to determine the parallelism of the slopes of dose-response curves for different congeners. The coefficient of determination (r^2) was used to determine which toxic endpoints for each congener in the ELS assay was the most consistent. The maximal EROD activity was determined by the Michaelis-Menton function for enzyme-substrate analysis. The statistical significance level was set at $p \leq 0.05$ for all tests. The corresponding TEF for congeners were calculated as the ratio of TCDD LC_{50} to congener's LC_{50}

RESULTS AND DISCUSSION

When medaka embryos were treated with various concentrations of TCDD, TCDF, PeCDD, or HeCDD, toxic effects were concentration dependent. Within Figure 1 is shown the concentration-dependent relationships for the appearance of any lesion, severe lesions, hatching inhibition and mortality. As the water concentration increased, the response of each endpoint also increased. The water concentrations were logarithmically transformed, and plotted against the responses on a probability scale. The resulting curve was fitted with a straight line by linear

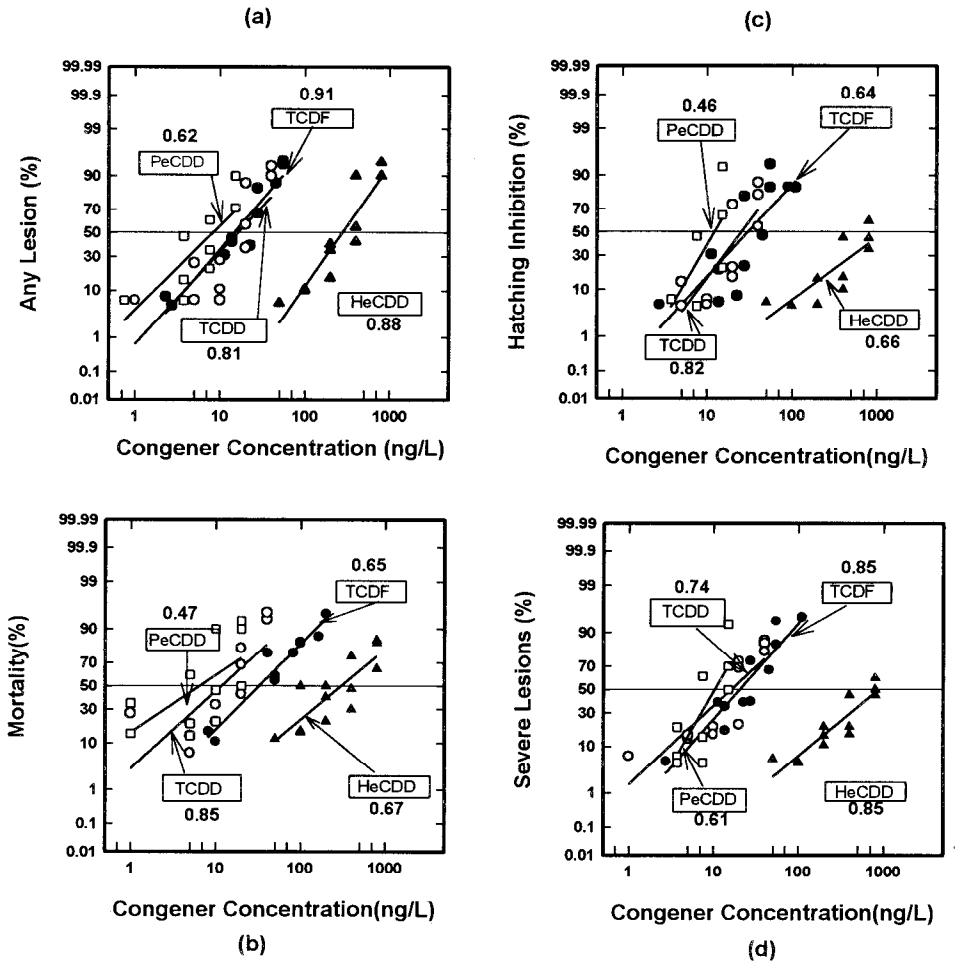

Figure 1. Toxic effects of different dioxin congeners on medaka embryos. (a) appearance of any lesion induced by dioxins; (b) 3-day post hatch mortality induced by dioxins; (c) hatching inhibition by dioxins; (d) appearance of severe lesions induced by dioxins. Numbers attached to the congener labels indicated the r^2 of the fitted dose-response lines on the logarithmic versus the Probit scales.

regression analysis. The coefficients of determination (r^2), as shown in Figure 1, of all lines indicated that the appearance of any lesion was the most reliable endpoint.

By using the EPA's Probit Analysis Program, we had determined various EC,, values, as well as the calculated TEFs, of different endpoints for all congeners tested as summarized in Table 1. The potency of each congener was as follows: PeCDD>TCDD=TCDF>>HeCDD. For the four toxic endpoints evaluated, the appearance of any lesion and the mortality were equally sensitive followed by the severe lesions and the hatching inhibition,

Wisk and Cooper (1990) described the appearance and the sequence of TCDD

induced lesions in detail. The same lesions were observed in this study, and were described as following. No lesions associated with TCDD toxicity were observed in the treated embryos until the formation of liver rudiment (day 5). Usually, but not always, the first observed lesion was the decreasing circulation in the caudal vein. The following sequence of lesions included hemorrhages or hemostasis in caudal, periorbital, vitelline veins and pectoral tin, and brain region. Pericardial edema was observed with hemorrhaging in different areas; however, both lesions were not always present concomitantly in affected embryos. Depending upon the severity of the lesions, these cardiovascular lesions were sometimes reversible. For instance, some embryos exhibited mild pericardial edema, but recovered without showing any sign of toxicity after 2 or 3 days. For some embryos, recurrences of lesions were also noted. It was also interesting to note that some animals exhibited severe edema after hatch with no lesions observed prior to hatching.

Table 1. EC_{50}s (ng/L) and TEFs (in parenthesis) of four dioxin congeners for different toxic endpoints observed in treated medaka embryos. The EC_{50} was calculated by EPA's Probit Analysis Program or by the Michaelis-Menton equation. TEF of each congeners=TCDD's EC_{50}/congener's EC_{50} TEF for TCDD was 1.00. NA: not available.

Congener	Any Lesion	Severe Lesions	Hatching Inhibition	3-day Post-hatch Mortality	EROD
TCDD	15.8 (1.00)	18.4 (1.00)	26.8 (1.00)	13.5 (1.00)	1.2 (1.00)
PeCDD	7.1 (2.20)	10.1 (1.80)	12.2 (2.20)	4.4 (3.10)	NA
TCDF	15.5 (1.02)	21.6 (0.85)	29.5 (0.91)	9.3 (1.45)	2.6 (0.48)
HeCDD	287.8 (0.06)	667.6 (0.03)	745.6 (0.04)	294.9 (0.05)	6.4 (0. 18)

Two other lesions that were observed and not previously reported included: the formation of a colorless spleen or decoloration of the spleen in the embryo. The spleen lesions were not dose-dependent (data not shown) and only occurred late in development (just prior to or after hatch). This was not observed in the control animals. Early hatching induced by TCDD and other congeners was also observed.

For each congener tested, EROD activity in each test was converted to a relative activity based on the control group basal activity. The EROD activity was increased in the larvae exposed to the higher concentrations of each congener. There was a maximal induction for each congener. At the much higher concentrations, the EROD activity decreased below the maximal induction. Figure 2 demonstrates these dose-response patterns for each congener. By using the Michaelis-Menton model for analyzing enzyme kinetic in a specific dose range, the maximal relative EROD activity induced by congeners were as follows: 19.7 for TCDD, 16.0 for TCDF, and 10.9 for HeCDD. Although the maximal EROD activity induced by PeCDD could not be determined due to large variations within two separated tests, it should be noted that, in one test, this congener could be induced up to 40 fold of

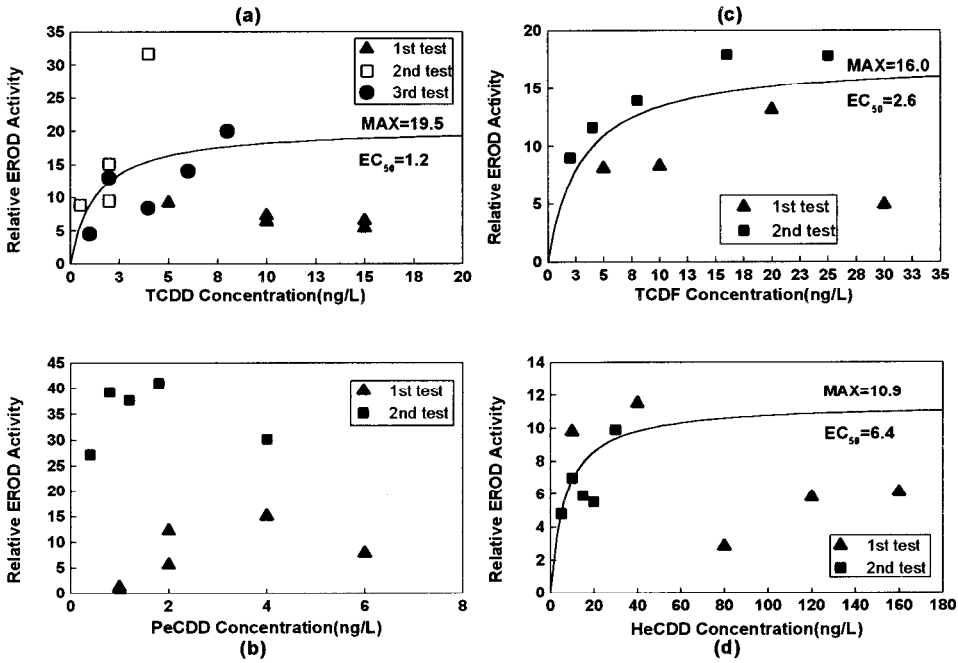

Figure 2. Concentration-response relationship of dioxins for induction of relative EROD activity (observed activity/basal activity in control) in treated medaka larvae. The maximal (MAX) activity was estimated by the Michaelis-Menten equation without using the data point showing decreased activity at higher concentrations. The effective concentrations for induction of 50% of the maximal activity (EC,,) were also shown. (a) 2,3,7,8-TCDD; (b) 1,2,3,7,8-PeCDD; (c) 2,3,7,8-TCDF; (d) 1,2,3,4, 7,8-HeCDD.

the basal activity (Figure 2b). The approximate concentration corresponding to 50% of the maximal induced EROD activities for each congener was estimated as follows: 1.2 ng/L for TCDD, 2.6 ng/L for TCDF, and 6.4 ng/L for HeCDD. These gave the TEF for TCDF of 0.48 and for HeCDD of 0.18, respectively.

Lesions caused by the different congeners were similar in the treated embryos, and resembled those described by Wisk and Cooper (1990). These lesions have also been reported in other fish species (Spitsbergen *et al.* 1991, Walker *et al.* 1992). In this study, 1,2,3,7,8-PeCDD was the most toxic among the four congeners tested with the TEF greater than 2 for different endpoints in the ELS assay. This has not been reported previously by other researchers for PeCDD. The 2,3,7,8-TCDF was about equally potent to 2,3,7,8-TCDD in inducing lesions, as well as other toxic effects, in the embryos. This is in comparison to a TEF of 0.028 for 2,3,7,8-TCDF in rainbow trout ELS mortality (Walker and Peterson 1991), and the proposed TEF of 0.1 for mammalian risk assessment (Safe 1990). Therefore, there is at least a IO-fold difference in medaka's sensitivity to this compound. The TEFs for 1,2,3,4,7,8-HeCDD ranged from 0.03 to 0.06, and were about 10 fold less than that proposed by Walker and Peterson (0.3) (1991).

Medaka larvae were exposed to various concentrations of different congeners to determine the concentration-response relationship for EROD induction. The basal activity in the larvae varied in each test. These values ranged from 1.55 to 8.6 pmol/min/mg of protein with the average of 3.9 pmol/min/mg (data not shown). This variation may reflect the increasing enzyme activity of the growing larvae, since larvae 3 to 10 days post-hatch were used. It has been reported that in sac fry and juvenile fish EROD activity increases rapidly at hatch and continues to increase as the larvae grow (Binder and Stegeman 1984). The increasing of benzo(*a*)pyrene hydroxylase (B(*a*)PH) activity in the developing medaka embryos, treated with or without TCDD, was demonstrated by Wisk and Cooper (1992). When the relative EROD activity for each congener was plotted against the exposure concentrations (Figure 2), the dose-response curves were similar to other studies using cultured chicken hepatocytes (Kennedy *et al.* 1992) or human squamous cell carcinoma (SCC) cell lines (Hudson *et al.* 1983). In both our studies and those reported in the literature, EROD or ECOD (7-ethoxycoumarin *O*-deethylase) activity reached a maximum followed by a decline at the higher congener concentrations. The mechanism for this inhibition/inactivation of EROD activity is unknown. However, in our studies cytotoxicity occurred at the higher exposure level which could explain the decreased enzymatic activity.

The relative potency for EROD induction was TCDD > TCDF > HeCDD (Table 1). Although the $EC_{50}$of PeCDD could not be determined, its EROD induction up to 40 folds of the basal activity was noted. The result of TCDF was different from that in the medaka ELS assay. The TEF of TCDF derived from the EROD assay was 0.48, while it was 1.02 from the ELS assay, and did not agree with TEF of 0.028 proposed by Walker and Peterson (1991) either. For HeCDD, the TEF of 0.18 was close to 0.3, which was proposed by Walker and Peterson (1991).

Medaka is a sensitive species to dioxins, especially for the developing embryos. A TCDD water concentration as low as 1 ng/L could cause cardiovascular lesions in exposed medaka embryos in this study. For medaka larvae, a TCDD concentration of 0.5 ng/L in water could result in a significant EROD induction (10-fold higher than the control group).

Some of the TEFs derived from this study do not agree with other studies. This, again, indicates that different systems or animals exhibit different susceptibility toward TCDD and its congeners. In addition, the potency of different congeners may not be similar in different assay in the same animal tested, resulting in deviation of calculated TEFs. However, the Japanese medaka ELS and EROD assays can be conveniently used to further determine the toxicity of a variety of environmental samples, such as sediments, and complex mixtures.

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