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Evaluation of PCDD/F Toxicity in Fish Livers from Ya-Er Lake, China: Chemical Analysis Compared with In Vivo and In Vitro EROD Bioassays

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Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) are found in worldwide environment. PCDD/F are formed as by-products in the production processes of industrial chemicals. Combustion, and pulp industry are also the sources of these compounds (Safe, 1990). Because of their lipophilic, chemically stable and toxical properties, these environmental contaminants may elicit toxic effects to organisms at the top of the food chains by biological accumulation (Wu, 1999).

The characteristic effects caused by 2,3,7,8-TCDD, the most potent congener among these compounds, include edema, immuno-suppression, hormonal alterations, monooxygenase enzyme induction (methyl cholantrene type), fetotoxicity, teratogenicity and carcinogenicity in laboratory animals. 2,3,7,8-TCDD is considered as a non-genotoxic carcinogen because DNA adducts have not been discovered and many in vitro assays for genotoxicity have given negative results (Kopponen et al., 1994). PCDD/F are approximate isostereomers, and probably exert most of their toxic effects with different potencies through the same biological receptor (Landers and Bunce, 1991). One of the most sensitive biochemical response is the induction of specific cytochrome P450 1A1 by PCDD/F, which can be measured as ethoxyresorufin-O-deethylase (EROD) induction (Landers et al., 1991; Li et al., 1997). These correlations validate the use of enzyme induction in vitro or in vivo rapid and simple bioassays for assessing the amount of dioxin-like compounds in the environmental samples. The induction of CYP1A1 by TCDD may also have profound effects on the carcinogenicity of other chemical through metabolic activation (Pohl and Fouts, 1980; Deml et al., 1989; Nebert, 1989).

The toxicity assessment of mixtures of PCDD and PCDF is based on the 2,3,7,8-TCDD toxicity equivalent (TEQ) which are calculated from the levels of the 17 PCDD/F congeners with 2,3,7,8-chlorine substitution (Safe, 1990, Ahlborg et al., 1994). At present, gas chromatography combined with high resolution mass spectrometry (HRGC/HRMS) is the standard method for quantitating PCDD/F, in which each individual congener has to be separated and quantitated. In practice, this is expensive and time consuming, even if only the 17 toxic PCDD/F congeners are measured. Moreover, toxic effects of mixed PCDD/F are not accounted. Therefore,

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short-term bioassays may serve as a useful tool in assessing the toxicity of complex environmental samples. In aquatic system, many investigations have been performed to monitor P450 1A1 activity in fish liver (Payne et al., 1987; Livingstone, 1993). Up to now, numerous studies have shown that CYP1A1 induction in fish liver is significantly related to PCDD/F levels in aquatic environment, and induction of CYP1A1 was found to be very sensitive (Payne et al., 1987; Gorge et al., 1992; Thomas and Karl, 1995). However the vital limitation of this in vivo bioassay is that other chemicals could also be inducers of P4501A1. In addition, the expression of P450 1A1 activity in fish can be affected by many factors, such as species, sex, age, reproductive stages, temperature, and possible inhibitors, different treatment of samples (Ankley et al., 1985; Vindimian et al., 1991). As an in vitro system, CYP1A1 inducible cell cultures hold considerable promise for toxicity assessment. Inducing toxicants can be extracted from environmental samples (e.g., sediments, biota, eggs of birds, etc.), and the extent to which they induce CYP1A1 in the cell cultures can be assessed as compared to the TCDD standard (so called toxic equivalent factor, TEF). An interesting development in this field is the micro-EROD assay for determination of enzymatic activities in intact rat hepatocyte cells with 96-well plates, requiring no cell disruption and allowing repeated assays with the same cell monolayer (Donato et al., 1993; Schwirzer et al., 1998).

Ya-Er Lake is located in the eastern part of Wuhan, China, and has been heavily polluted by the effluent of chloroalkali industry. In 1978, the Ya-Er Lake was divided into a series of five ponds for self-purification, among which Pond 1 is the nearest site to the effluent and the Pond 5 is the furthest. In recent years, high levels of PCDD/F were found in sediments of Ya-Er Lake, among which sediment in Pond 1 is the most polluted site with PCDD/F concentration high to 177ug/kg (I-TEQ, 800ng/kg) (Wu et al., 1998a). The present study describes the comparison of in vivo, in vitro, and chemical results of P450 1A1 in fish livers from the Ya-Er Lake.

MATERIALS AND METHODS

Crucial carp exhibiting normal behavior were captured by using fishing net from five ponds of Ya-Er lake. During sampling time, the temperature in Ya-Er Lake was about 15±2 °C. Eight crucial carps about 2 to 3 years old were selected in each pond and killed immediately. A small part of livers were excised and stored respectively in liquid nitrogen for the in vivo bioassay. The remaining part of livers were freeze-dried (Christ Alpha Company, Germany) and pulverized (Retsch Company, Germany) for in vitro bioassays and chemical analysis. Among these samples, there were only seven fish which liver size was big enough for chemical analysis and bioassays performed. The livers of other fish in each pond were mixed.

About 1-5g aliquot of dry fish liver was soxhlet-extracted using 180 ml toluene for 24h. For chemical analysis, since the process is reliable and expensive, no replicates has been performed. The samples were spiked with $^{13}C_{12}$ labelled 2,3,7,8-substituted

PCDD/F prior to extraction (isotope dilution method). Cleanup of samples achieved by several liquid chromatographic purification steps including aluminum column, mixed silica column and florisil column. 1,2,3,4-$^{13}C_{12}$ TCDD was added last as recovery standard for HRGC/HRMS analysis. The clean-up and quantification were carried out as described elsewhere (Henkelmann et al., 1996; Wu et al., 1998b). Mass spectrometry (MS) measurements were conducted using a Finnigan MAT95s (R = 10000) instrument for isomer specific measurement.

For micro-EROD bioassay in vitro, replicate extractions were performed in each sample. About 1-5g dry fish liver was soxhlet-extracted using 180 ml toluene for 24h, without addition of the PCDD/F internal standard. Concentrated liver extract from soxhlet-extraction was applied to a column filled with n-hexane. The column was packed with 10 g silica (44% conc. sulphuric acid, w/w), 40 g de-activated silica (4% water w/w) from bottom to top. The silica type was active and 63-200 μm. The column was topped with anhydrous Na_2SO_4. Samples were eluted by 870 ml n-hexane and the elute was evaporated (559 mbar, 343 K) to 2-3 ml. The extract was stepwise transferred into a micro-vial and evaporated to dryness under a steam of nitrogen. The sample was re-dissolved with 500 μl of DMSO/isopropanol (4:1, v/v).

Rat hepatoma cells H-4-II E C3/T (H-4-II E) were grown in Dulbecco Minimum Essential Medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 95% relative humidity, 37 °C, and air containing 5% CO_2 (Deml et al., 1989). The EROD activity in intact cells seeded on 96-well plates was determined according to Wu et al. (1997) modified as follows: cells were seeded at a density of 1 x 10^4 / well in a 96-well plate. After 3 days the medium was replaced with 100μl medium containing 0.5μl sample extract or TCDD standards (Sigma Company) which were dissolved in DMSO/isopropanol. Each extract and standard was performed triplicate. After 24h, 48h, and 72h the medium was removed from different plates and 100μl fresh medium containing 8μmol 7-ethoxyresorufin and 10μmol dicumarol were added. After incubation for 60 min at 37°C, 90μl medium was transferred to a fresh 96-well plate and 130μl methanol was added. Resorufin-associated fluorescence was measured in the solutions on a multiwell fluorescence reader (Fa. Fluoroskan11, Fa, Labsystem, Australia). The protein content per well was assayed using bicinchoninic acid according to Smith et al. (1985). Standard deviations between triplicates remained less than 10%. TCDD standard curves were run with every set of samples to calculate TCDD toxicity. The biological TEQ values were calculated according to Hanberg et al. (1991) comparing the induction of enzyme activity by environmental sample extracts with the series concentrations of TCDD standard. In order to obtain reliable bioassay results, sample extracts were usually diluted to different concentrations until the response could be found in the effective region of standard curve. Results derived from the standard curve should be related to original sample concentration by back calculation using the proper dilution and volume factors.

For in vivo EROD assay, enzyme preparation is the first step. Approximate 0.1g fish liver from liquid nitrogen was placed in a glass homogenizer with 1.0ml iced

phosphate buffer PBS (pH=7.6) and homogenized for 2 min. The homogenates were centrifuged at 10,000g for 10min in a centrifuge (Hettich Company, Germany). The supernatants were used for EROD assay and protein content determination. EROD activity measurements were made by spectrofluorometic detection of the end product resorufin, after oxygen deethylation to calculate the enzyme activity (Li et al., 1997). The spectrofluorometer was calibrated by a series of resorufin standards (0-250 nM). The assaying mixture consisted of 1.98 ml phosphate buffer (PBS, pH 7.8), 10μl of 0.2 mM ethoxyresorufin, 10μl prepared supernatant sample. After mixing the above reagents thoroughly for six minutes, the reaction was initiated by adding 10 μl of 6 mM NADPH. The negative controls were performed without NADPH. The reaction was controlled at the temperature of 20 ± 1 °C and stopped by adding 0.5 ml methanol after 10 min. The measurement of each sample was carried out on Kontron SFM 25 spectrofluorometer (Sweden) at excitation wavelength 560 nm and emission wavelength 580 nm. Each induction of EROD activity was the mean value from five measurements.

RESULTS AND DISCUSSION

For determination of EROD-TEQ in unknown samples, a standard curve used which is based on the induction value of 2,3,7,8-TCDD standards with intact H4IIE cells in 96-well plates (Tillitt et al., 1991). Figure 1 shows the resulting standard curve for TCDD with micro-EROD assay in present study. The EC50 is 0.9 pg /ml TCDD, and the standard deviation is no more than 5%. The detection limit in intact H4IIE cells is 0.4 pg /ml TCDD, which equals an absolute amount of 0.04pg /well TCDD in 96-well plates. Recovery levels for the extraction and clean-up of samples for the chemical analysis were ranged from 85% to 115%.

Table 1 summaries the results of EROD bioassays in vivo and in vitro and chemical nalysis in 7 bigger fish livers. The biodegradation or biotransformation of xenobiotics is a complex process, and the expression of P450 1A1 activity can be affected by many factors. In order to confirm the accurancy of in vitro EROD assay to evaluate the toxic effect in fish, the micro-EROD TEQs were compared with the analytical TEQs measured in the same fish liver sample. Equation1 and equation2 show the correlations between micro-EROD TEQs and chemical TEQs in fish livers. It can be concluded that although EROD TEQs from 24h exposure are higher than the EROD TEQs from 72h exposure, which is due to the metabolism of dioxin-like compounds (e.g. PAHs) existing in extracts, both EROD TEQs are comparable to analytical TEQs ($R^2 > 0.95$). The big differences of concentrations are obvious in fish livers from the different sampling sites along the effluent flow direction, but the results between chemical analysis and in vitro bioassay are still well correlated. For most of fish livers the EROD TEQs (from 72h exposure) are slightly higher than the values from chemical analysis (Table 1). This is due to the fact that bioassay measures the responses to all potential toxic compounds which are able to bind to Ah receptor and thereby induce CYP1A1 in fish liver. Thus, The micro-EROD assay represents an intergrated measurement of the toxicity of the extract. The comparative results give a further confirmation that the micro-EROD assay is

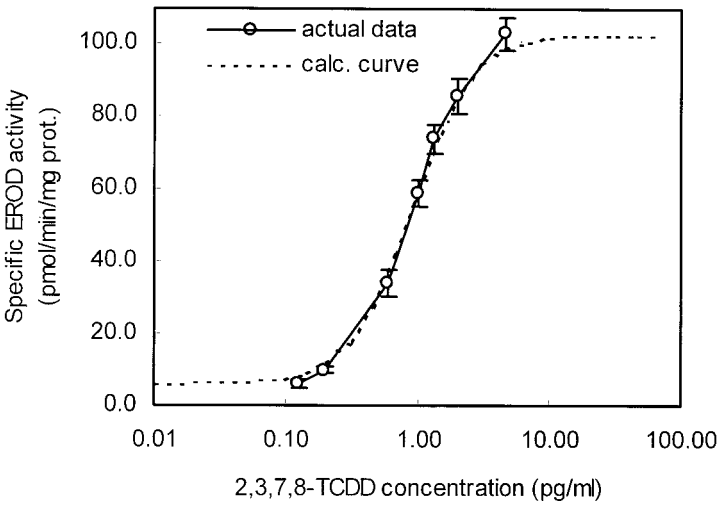

Figure 1. Dose-response relationship for 2,3,7,8-TCDD with micro-EROD-assay in intact rat hepatocytes with 96-well cell culture plates. Cells were exposured to 2,3,7,8-TCDD in DMSO:isopropanol (0.5% v/v, in medium) for 72h. Error bars represent the standard deviations with n=4.

reliable for the determination of TEQ in fish livers, if the single clean-up step has efficiently removed the interferences of bioassay from fish liver.

$$Y = 1.07X + 47.25 \quad (R^2 = 0.9779) \tag{1}$$

Where: X: Chemical TEQs
Y: In vitro EROD TEQ (24h)

$$Y = 0.86X + 19.55 \quad (R^2 = 0.9542) \tag{2}$$

Where: X: Chemical TEQs
Y: In vitro EROD TEQ (72h)

$$Y = 3.75X + 50.68 \quad (R^2 = 0.9773) \tag{3}$$

Where: X: In vivo EROD activity
Y: In vitro EROD TEQ (24h)

$$Y = 3.02X + 22.48 \quad (R^2 = 0.9505) \tag{4}$$

Where: X: In vivo EROD activity
Y: In vitro EROD TEQ (72h)

$$Y = 3.44X + 4.79 \quad (R^2 = 0.9722) \tag{5}$$

Where: X: In vitro EROD activity
Y: Chemical TEQ

Table 1. The results of EROD bioassays in vivo and in vitro and chemical analysisin 7 bigger fish livers

Sample	Site	Chemical TEQ (ng/kg liver)	In vitro EROD TEQ (ng/kg liver) (n= 6)		In vivo EROD activity (n=5) (pmol RF/mg protein min)
			24h	72h	
Fish 1	Pond 4	4.33	30±2.98	18±1.61	0.88 ± 0.05
Fish 2	Pond 4	8.25	57±3.51	20±2.24	5.23 ± 0.30
Fish 3	Pond 3	15.5	64±3.44	30±2.21	6.26 ± 0.39
Fish 4	Pond 3	23	117±8.91	50±3.55	11.4 ± 1.08
Fish 5	Pond 2	111	140±2.98	105±6.45	14.6 ± 0.87
Fish 6	Pond 1	300	353±17.7	336±17.8	88.8 ± 4.52
Fish 7	Pond 1	379	473±22.5	306±18.5	107 ± 9.94

Equation 3 and Equation 4 show that the results of in vitro EROD assay are significantly correlated with the results of in vivo EROD assay ($R^2 > 0.95$). It indicaets that in vivo EROD assay is comparable with in vitro assay. It implies that in vivo EROD assay may be used as a biomarker as in vitro EROD assay to evaluate dioxin exposure in Ya-Er Lake which is polluted by chloralkali effluent.

Because the samples for the in vivo bioassay were collected and stored in liquid nitrogen in situ, the results obtained can reflect the toxic effect of actual dioxin exposure in water. However the vital limitation of in vivo bioassay is that other chemicals could also be inducers of P450 1A1. Hence, in vivo bioassay has higher requirements for the sample type (must collect from living animals) and sample storage; samples have to be measured within a short time. But in vitro bioassay has less requirements for sample storage and measuring time, and it can be employed in any kind of samples. Equation 5 shows that the results between in vivo bioassay and chemical analysis are comparable ($R^2 = 0.9722$). It indicates that the EROD induction in fish liver can reflect the toxic effect of long-term dioxin exposure in water.

Figure 2 shows the results of in vitro and in vivo bioassays and chemical analysis of fish livers in different sampling sites along the effluent flow direction (from Pond1 to Pond 4). It is obvious that the results obtained from these three methods have the same trend. Along the effluent flow direction, the results are downgraded, which are consistent with the concentrations of PCDD/F in sediments of these four ponds (Wu et al., 1998a). In vitro micro-EROD bioassay, although there are differences between the results from 24h, 48h and 72h exposure, their trends are still consistent. However, the micro-EROD TEQs from 72h exposure are more close to the chemical TEQs than that from 24h and 48h exposure. The results show that in vitro micro-EROD assay, 72h exposure is necessary and enough to efficiently metabolize some non-persistent CYP1A1 inducers.

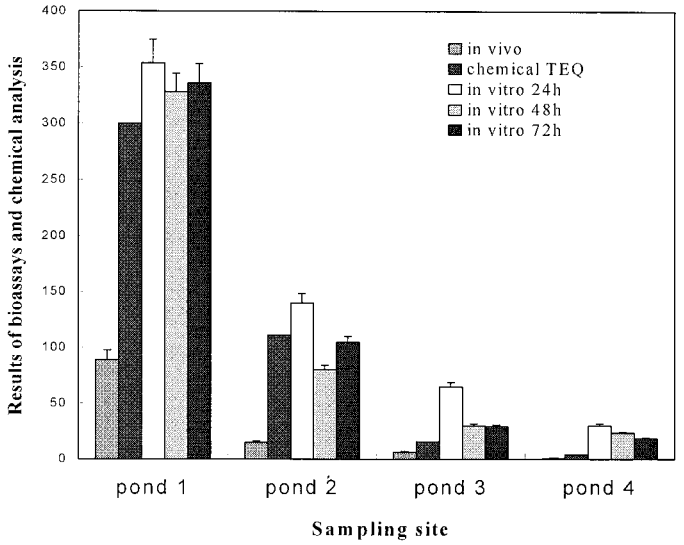

Figure 2. The results of in vitro, in vivo bioassays and chemical analysis of fish liver in different sampling sites along the effluent flow direction (from Pond1 to Pond 4). The data for the in vivo assay used different units (i.e pmol/mg min) than the chemical data and in vitro data (i.e. ng/kg). Error bars represent the standard deviations with n=7.

Our results showed that the micro-EROD-assay is a rapid and sensitive method to determine PCDD/F toxic potency in intact H4IIE hepatocyte cells in 96-well plates. The good correlation between the results from micro-EROD and chemical analysis confirms that the micro-EROD-assay with its special cleanup is sufficiently reliable for the determination of TEQ in fish livers. The comparative results of EROD bioassay in vivo to chemical analysis and to micro-EROD bioassay in vitro show that the EROD induction in fish liver is useful for indicating the in situ toxicity of PCDD/F exposure in aquatic ecosystem. The EROD bioassay in vivo and vitro can be complementary with each other for rapid screening in toxicity assessment.

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