Toxic effects of 2,3,7,8-tetrachlorinated dibenzo-p-dioxin (TCDD) during a different life stage test with Chinese Rare Minnow(*Gobiocypris rarus)*

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Introduction

Rare Minnow (*Gobiocypris rarus*) is a tiny carp, abundant in China. Many research[1,2] results have shown that this species possesses the characteristics of good life adaptability, short period for growing-up and sex mature. It is easy to be cultured in laboratory all the year round. Therefore, Rare Minnow is considered as an ideal fish species for ecotoxicological research in China. Several heavy metals and pentachlorophenol have been tested with it for the research of toxicology[3].

Due to the environmental persistence and potent biological properties, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans(PCDD/F) have attracted a great deal of attention and are listed as the important priority contaminants in developed countries. But in China, further studies on PCDD/F are restricted because of the high cost involved. It has been known that those compounds with 2,3,7,8-chlorine substitution, especially 2,3,7,8-tetrachlorinated dibenzo-p-dioxin(TCDD) being the most active. The intoxication mechanism is mediated through combining with the celluar Ah-receptor[4] followed by acting on the cytochrome P450 1A enzyme system. The induction of 7- ethoxyresorufin-O-deethylase (EROD) activity, the most sensitive biochemical response, has been used as a bioindicator for assessing TCDD toxicity[5,6]. But currently, the related reports about TCDD toxicity are mostly obtained from the short time exposure or only by one time injection mode. The information about the long-term toxic effects exposed to TCDD with low doses are very limited. Since PCDD/F are widely spread and can be found everywhere, humanbeing actually has long been exposed to these compounds included TCDD. It is recognized recently that the long-term toxic effects of the exposure to TCDD with low doses may probably be related with the reproductive toxicity of TCDD. Therefore,the aim of the study is: to determine the sensitivity of Rare Minnow to TCDD toxicity and assess the long-term toxic effects of TCDD with low doses in Rare Minnow using EROD activity induction as a bioindicator.

Materials and Methods

1. Chemicals

2,3,7,8-TCDD was synthesized by Wuhan University, and tested as 99% pure using HRGC/HRMS isotope dilution method. Except for 7-ethoxyresorufin, resorfin and NADPH were biochemicals purchased from Sigma Co., USA, the rest were all Chinese reagents with analytical purity.

2. Rare Minnow eggs

In this study, the newly fertilized Rare Minnow eggs (mean weight ca. 1.5mg) were obtained from the Institute of Hydrobiology. The hatched fry were fed with *Artemia salina* daily during the test period.

3. Exposure to 2,3,7,8-TCDD

The eggs were divided into the control groups containing 0.3ml acetone in 300ml water and the exposure groups consisting of 0.3ml acetone with different doses of TCDD in 300ml water. The concentrations of TCDD in exposure groups were: 0.002, 0.005, 0.01, 0.03, 0.05, 0.1, 1.0, 5.0, 10, 50, and 100 pg/ml. 200 eggs were placed in each group and hatched at 25±1^{0}C in a continuously aerating tap water system. The test solution in each group was replaced twice a day before hatching and once a day after hatching. The whole test period lasted 4 months. For each sampling, 50 eggs or embryos were collected daily and approximately 0.1g sac fry or fry was taken every 15 days for the analysis. Then the sample was homogenized in a glass-made container in 1.0ml ice cold phosphate buffer (0.125M $KH_2PO4/Na_2HPO_4.12H_2O$ + 0.05mM Na_2EDTA, PBS, pH 7.6). The homogenates were centrifuged at 10,000 rpm for 10 mins. Finally, the supernatants were used for the determination of EROD activity and protein.

4. EROD assay

The EROD assay was conducted by a modification of the method described by Pohl et al.[7]. The reaction time and substrate concentration of the incubation was optimized. The assaying mixture consists of 1.88ml phosphate buffer (PBS, pH 7.8), 10 μ l of 0.2mM ethoxyresorufin, 100 μ l of sample supernatant. After mixing above reagents thoroughly for 6 mins, the reaction was initiated by adding 10 μ l of 6mM NADPH. The incubation temperature was controlled at 20±1 ° C and stopped after 10 mins by 0.5ml ice-cold methanol. Each determination of EROD activity was carried out by measuring resorufin as the end product on a Kontron SFM 25 spectrofluorometer at excitation wavelength of 560nm and emission wavelength of 580nm. The protein was determined according to the method of Lowry et al.[8] using bovine serum as standard.

5. Examination under optical microscope

During the embryonic development, Rare Minnow eggs, sac fry and hatched fry were taken both from control groups and exposure groups for examining the effects caused by TCDD toxicity. The observations of pathologic alterations in each sample examined were recorded. The sac fry mortalities in the exposure groups were used for the calculation of NoEL (No Effect Level) and LoEL (Lowest Effect Level) of TCDD. The sex of mature Rare minnow was identified under optical microscope by the end of the test.

Results and discussions

1. Accuracy of EROD assay

Fig. 1 and Fig. 2 are the standard curves for resorufin and protein measurements. Their correlation coefficients were obtained as 0.9998 and 0.9991, which indicated that the good linearity and accuracy in the measuring ranges for EROD assay.

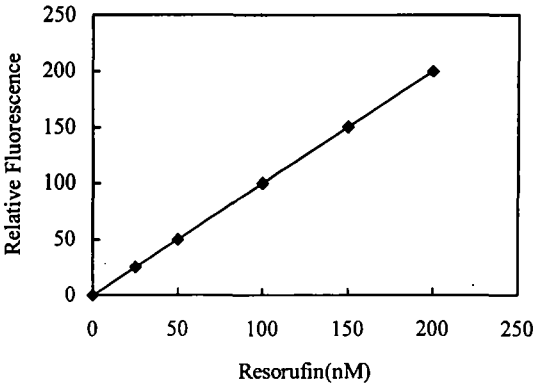

Fig. 1 Standard curve for resorufin analysis

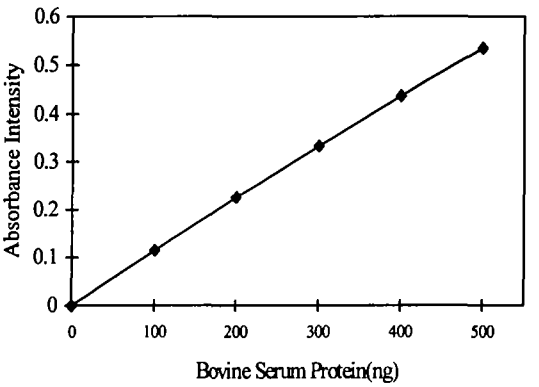

Fig. 2 Standard curve for protein analysis

2. Toxic effect during early life stage

During early life stage, the EROD activities in Rare Minnow eggs, embryos and sac fry were determined every day. Fig.3 showed the effects of TCDD concentrations in water on EROD activity induction after being exposed for 2, 3 and 5 days. It was found that EROD activities in fertilized eggs were induced after 2 day exposure at the concentrations of TCDD ranged from 0.1 to 100 pg/ml. In the exposure groups with less than 0.1pg/ml TCDD and control (TCDD-free) groups, no obvious difference of EROD activity was found between them. In the examination under optical microscope, it was observed that dose-related increases in sac fry mortality, yolk sac edema, hemorrhages and arrested development in the exposure groups with higher doses (≥1.0pg/ml)of TCDD during the early life stage of Rare Minnow. These toxic syndromes were very similar to blue sac disease, and finnally they all died within 15 days as seen that in Rainbow trout. In those groups with TCDD doses ranged from 0.1to 1.0 pg/ml, the hatched sac fry could be survival only for 15 to 30 days and finally also died of edema. But the hatched sac fry could be alive and grow up in the water containing TCDD concentrations less

than 0.1pg/ml. This concentration was just the lowest concentration of TCDD being able to induce EROD activity. It meant the sac fry mortality and pathologic alternation were consistent with the induction of EROD activity. So, a series of dilutions starting with the TCDD concentration of 0.1 pg/ml were prepared as the low doses for the following long-term exposure tests. Meanwhile, the TCDD toxicity in Rare Minnow during the early life stage was proved to be very sensitively indicated by the EROD activity induction.

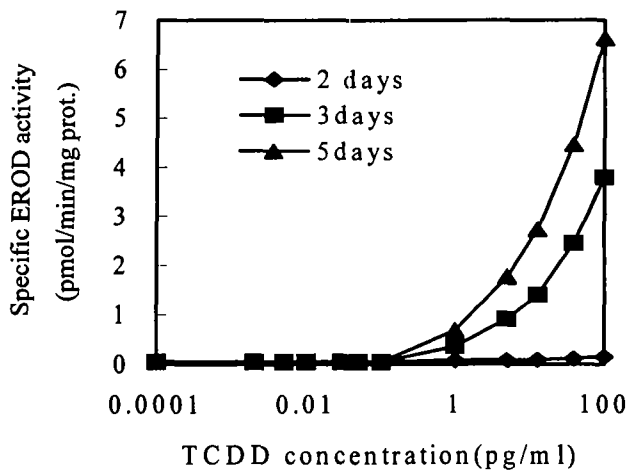

Fig. 3 Induction of EROD activity in Rare Minnow eggs during early life stage

3. Long-term effects of TCDD toxicity with low dose

Fig. 4 shows the time-response relationship between the induction of EROD activities and TCDD with low dose. From this time course of EROD activity induction in Rare Minnow, it can be clearly seen that the induction of EROD activity dramatically increased during the first month. But in the second month, the induction of EROD activities were getting less. In the last two months, the induced EROD activities tended to become relatively stable and the different maximums of EROD activities were reached in different dose groups. In fact, this trend reveals the bioaccumulation regularity of hydrophobic contaminants included TCDD. In Fig.5, the long-term exposure dose-response relationships between TCDD doses in water and EROD activity inductions were observed in different life stages. As seen in Fig 5, the EROD activities in Rare Minnow during different life stages were significantly correlated with the TCDD concentrations. The inductions of EROD activities in Rare Minnow increased with the increasement of TCDD concentrations. The relationships between EROD activities and TCDD concentrations in the last two months were close to overlapped. In addition, the longer time exposure displayed more induction of EROD activity for the same doses of TCDD exposure. This implys that long-term toxic effects caused by TCDD with low dose exposure may more serious than that caused in short period. At the end of the test, all the mature Rare Minnow were taken out from both control groups and exposure groups for identifying their sex. It was discovered: the ratios of female to male are 1.2:1 in control group and 4:1 in 0.05 pg/ml TCDD

exposure groups. These two ratios clearly indicated the femalization of Rare Minnow resulted from the long-term effects of TCDD toxicity.

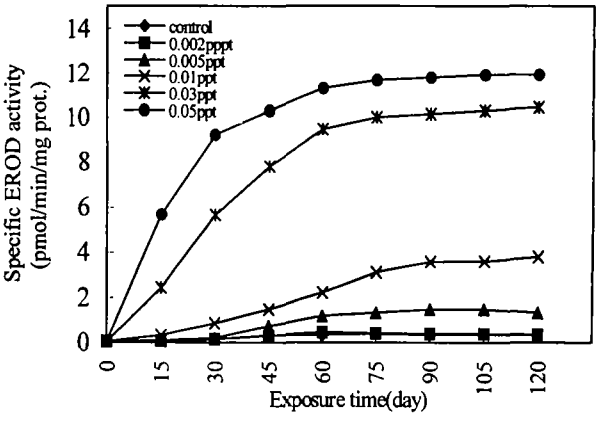

Fig. 4 The relationship of EROD activity and time in the long term exposure

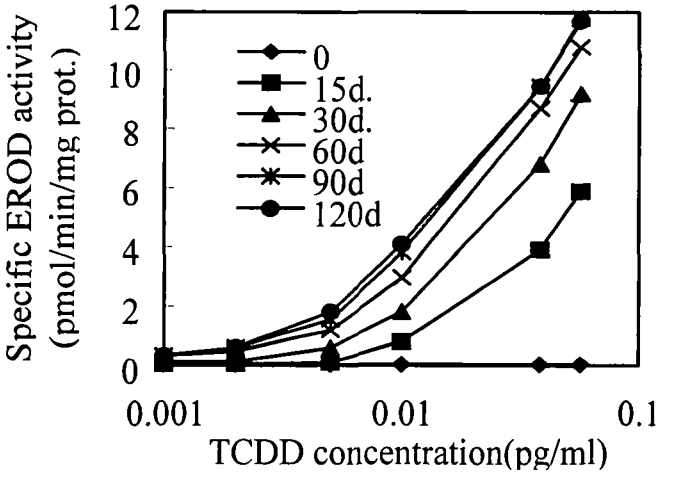

Fig. 5 The relationship between the EROD activity and TCDD concentration in different developmental stages of *Gobiocypris rarus*

Conclusion

From this study, conclusion can be drawn as following:

1.Rare Minnow is a species as sensitive as Rainbow trout to TCDD toxicity.

2.EROD activity can be induced in eggs, embryo, sac fry and mature Rare Minnow. The good response relationship between EROD activity and low dose TCDD exposure in water demonstrates that the long-term toxic effects of TCDD with low doses in Rare Minnow can be sensitively indicated by the EROD bioassay.

3.The time-related EROD activity induction reveals the bioaccmulation regularity of TCDD in Rare Minnow. Therefore, the established EROD bioassay could be developed to assess the bioaccmulation regularity of TCDD in aquatic environment.

4. The femalization of Rare Minnow resulted from long-term toxic effects of 2,3,7,8-TCDD with low dose shows that this chemical can act as an estrogen having reproductive toxicity.

Acknowledgments

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References

(1) Wang Jianwei, 1992, Reproductive biology of *Gobiocypris rarus*. Acta Hydrobiologica Sinica, 16, 2: 165-174

(2) Chang Jianbo, Wang Jianwei & Cao Wenxuan, 1995, The embryonic development of *Gobiocypris rarus*, Acta Hydrobiologica Sinica, 19, 2: 97-103

(3) Zhou, Y. X.; Chen, S. P. & Huang; W., 1995, Seven days subchronic toxic test on *Gobiocypris rarus*, Acta Scientiae Circumstantiae. 15, 3: 340-375

(4) Landers, J. P. and N. J. Brunce, 1991, Review article. The Ah receptor and the mechanism of dioxin toxicity. J. Biochem. 276, 273-287.

(5) Wu, W. Z.; Schwirzer, S. M. G.; Schramm, K. W.; Wiebel, F. J.; Xu, Y.; Zhang, Y. Y.; Yediler,A. & Kettrup, A. 1996, Rapid bioassay as indicator of potential harmful effects for dioxin-like compounds in the samples from Ya-Er lake,China.Fresenius Environ. Bull. 5:374-379

(6) van der Weiden, M. E. J., J. van der Kolk, R. Bleumink, W. Seinen and M. van der Berg, 1992, Concurrence of P4501A1 induction and toxic effects after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the Rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology, 24: 123-142.

(7) Pohl, R. J. and J. R. Fouts, 1980, A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fraction. Anal. Biochem., 107, 150-155.

(8) Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951, Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

(9) Xu, Y.; Wu, W. Z. & Zhang, Y. Y., 1996. Rapid quantitative screening of dioxin-like compound by EROD induction bioassay. China Environmental Science, 16, 4: 106-111.