Bull. Environ. Contam. Toxicol. (2004) 73:153–160

DOI: 10.1007/s00128-004-0407-z

Bulletin of Environmental Contamination and Toxicology

Immunotoxicity of Pentachlorophenol on Macrophage Immunity and IgM Secretion of the Crucian Carp (*Carassius auratus*)

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Received: 28 July 2003/Accepted: 21 April 2004

Pentachlorophenol (PCP), an organic chlorophenol, is widely used as industrial raw material, pesticide and wood preservative. Consequently, the heavy use of PCP pollutes the aquatic environment. The average sediment concentration of PCP in the middle and lower reaches of the Yangtse River, China is 173 ng/g (Martens et al. 2000). However, the maximum ingestible dose of PCP for safety is only 0.5 μg/L by FAO (1991) and 1 μg/L by Agency for Toxic Substances and Disease Registry (2001), United States, respectively.

PCP is considered to be a highly toxic contaminant. It can contaminate water and be accumulated rapidly in fish and shrimp and thus cause death (Roszell and Anderson 1996). Recent research has also shown that PCP is a suspected environmental endocrine-disrupting chemical (EED). As is well known, EED can suppress the immunocompetence of organisms. However, previous studies mainly examined the immunotoxicological and biochemical mechanisms of PCP on mammals (Blakley et al. 1998). Studies on fish macrophage function were conducted only with ocean fish (Roszell and Anderson 1997; Karrow et al. 1999). Few data can be found to assess the effect of PCP on macrophage immune function or on humoural immunity of freshwater fish. This study examined the immunotoxicity of PCP to crucian carp, one of China's main economic fish species, in in vivo and in vitro tests.

MATERIALS AND METHODS

Crucian carp (*Carassius auratus*) (< 1 year of age, average body weight 329.0 ± 20.69 g, length 25.5 ± 6.4 cm) were obtained from Jiangsu Aquatic Cultivation Center (Nanjing, P R China) and maintained at 14 ± 1℃, using a 12 hr light and 12 hr dark cycle in fresh chlorine-free water for 8 d before use. PCP and FITC-conjugated dextran FD-40s were obtained from Sigma Chemical (St. Louis, MO, USA). Percoll gradient (original density 1.18g/ml) and Leibowitzs L-15 medium were obtained from Pharmacia Corporation (Peapack, NJ, USA).

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Phorbol myristate acetate (PMA) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Luminol was obtained from MatTek Corporation (Ashland, MA, USA). Biotinized anti-carp IgM Rabbit IgG was prepared in our lab (Chen et al. 2003). All test chemicals and reagents had a purity of 99% or greater.

Macrophages were isolated by a modification of the method described by Roszell and Anderson (1996). In brief, three or four fishes were selected randomly and sacrificed. Pronephroi of all the fish were removed and abraded in a tissue grinder with L-15 medium. The mixture was filtered twice and layered gently into 34/51% percoll, centrifuged at 4℃, 400×g for 25min. The cells were collected from the interface of the suspension and the upper layer of percoll and washed twice using 5ml L-15 with 1% antibiotic solution (10,000 U/ml penicillin, 20 mg/ml streptomycin). To obtain purified macrophages, the cells were transferred into a Nunclon 6-well microtiter plate (Delta Inc. Denmark) and incubated at 17℃ for 2 hr in the plate. The supernatant was discarded and the adhered cells were then harvested as macrophages. The macrophages were resuspended with 3ml of L-15 medium. Quantification of surviving cells was determined by the exclusion dye trypan blue. The shapes of macrophages were photographed to determine cell type. Cells were adjusted to a final concentration of 2×10^6 cells/ml.

Macrophage viability was determined based on reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Macrophage suspension and various concentrations (0.5, 5, 50, 500 mg/L) of PCP were introduced into a 96-well microplate and incubated at 17℃ for 4 hr. The supernatant was then discarded and the cells were resuspended with 90 μL L-15. 10 μL MTT was added into each well and incubated at 17℃ for 4 hr. At the end of the reaction, 100 μL DMSO was added to dissolve the sediments. 20 min later, the optical density of the mixture was measured at 570nm using a Bio-Rad 550 microplate reader. L-15 + macrophages and L-15 were used as the negative control and blank. Each test had 8 replicates.

Determination of reactive oxygen intermediates (ROIs) was performed by the optimized method of Zhu et al. (1999). For each sample, 1 mL of cell suspension following PCP exposure was added into the test tube and 10 μL Luminol was added into each tube. The self-fluorescence of unstimulated cells was detected by a TD20-20 Luminometer (Avocado USA) for 1 min. PMA was added at 200 mg/L. 0.5 ml Luminol was then added. Chemiluminescence was measured at a one-min interval from 1 to 3 min. Each test was carried out in 8 replicates and relative luminescence units (RLU) were expressed as the difference of the average fluorescence between unstimulated cells (cells and medium only) and stimulated cells (cells, medium and PMA).

Phagocytosis was measured according to modified method of Piemonti et al.

(2000). In brief, 500 μL of exposed cells and 100 μL FITC-dextran were put into each tube successively, and incubated at 17℃ for 1 hr. After incubation, cells were centrifuged at 100 × g for 8 min at 4℃. The cells were washed twice by ice-cold PBS to stop phagocytosis and remove unphagocytized dextrans. The fluorescence of phagocytized dextrans was determined by a FACSCalibur flow cytometer (Beckson Dikinson Inc.). For each sample, 10,000 events were acquired and results were expressed by subtracting the mean fluorescence of the control from mean fluorescence of the sample.

In vivo exposure was performed by dividing fish into 4 groups, with 3 fish in each. Each group was maintained at room temperature (20-22℃) in 40 L fresh chlorine-free water (pH 7.4, dissolved oxygen 7.9 mg/L, alkalinity 2.5 m*M*, hardness 2.2 m*M*) in a 50 L tank. Exposure concentrations of PCP were 0.027, 0.053, 0.13 mg/L, respectively. The control group was given an equivalent amount of deionized water. The exposure lasted for 14 d, and half of the water was changed every 24 hr. Following exposure, macrophages were isolated and purified using the same method as the in vitro test. Phagocytosis was also tested using the same method of the in vitro test. The experiment was replicated with the same conditions.

Serum IgM levels were tested by the following method. Biotinized anti-carp rabbit IgG was dissolved in Na_2CO_3-$NaHCO_3$ (0.05 mol/L, pH 9.5) as a coating solution. A 96-well microplate was coated at 50 μl of the coating solution per well and incubated overnight at 4℃, then rinsed four times by PBS-Tween (0.01 mol/L, pH7.4). 200 μl block solution (PBS including 5% bovine serum) was added into each well. 100 μl fish serum, diluted 10^6 folds, 100 μl of 300 ng/mL biotinized rabbit IgG and 100 μl ABC Kit (Vector, USA) were added into each well successively and incubated together at 37℃ for 1 hr. Then 100 μl OPD-H_2O_2 was added. 5-10 min later, 50 μl H_2SO_4 (2 mol/L) was added to stop the reaction. Optical density was determined at 470 nm by a Bio-Rad 550 microplate reader.

Changes in the mean value of each parameter involved in the test were performed as a one-way unvariated and repeated Student's T-test, using the Origin software package, with repeated measurement for statistical significance. Parameters measured in each test group were compared to those of the control group. Groups with a P value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cells could be seen clearly through an optical microscope (Figure 1). Cell survival was calculated by dividing the number of total cells by that of viable cells, and the result was >99.1%. Impact of PCP on cell viability can be seen in Figure 2. Cell survival rate of macrophages at the low PCP concentration (0.5 mg/ml) was

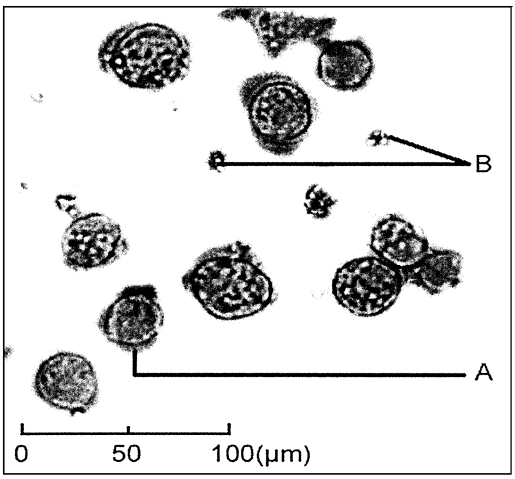

Figure 1. Shapes of macrophages via microscopy
A: Macrophages B: Red Blood Cells

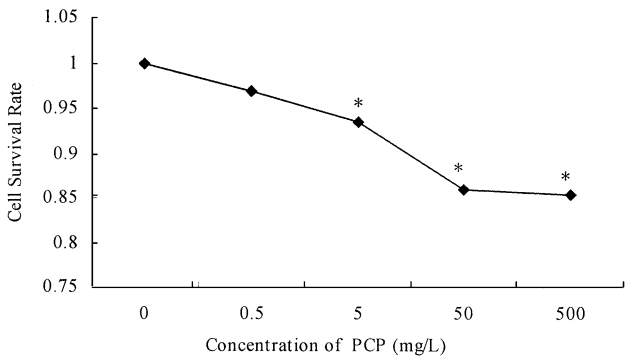

Figure 2. Change of in vitro viability in crucian carp macrophages
* Significant difference versus control group ($P<0.05$)

96.8%; i.e., it was not significantly suppressed ($P>0.05$), while the other experimental groups were significantly suppressed. Cell survival rates of macrophages at the concentration of 5 mg/L, 50 mg/L and 500 mg/L PCP were 93.4%, 85.9% and 85.3% respectively. Statistical analysis showed that the cell number reduction was in a PCP-dose-dependent pattern.

Figure 3 shows ROIs production after exposure to PCP. The columns of each concentration in the figure represent the RLU after 1, 2 and 3 min of the activity. RLU after 1 min of respiratory burst were 0.26, 0.032, 0.065, 0.059 and 0.036, respectively ($P<0.05$); mean RLU of 2 min were 0.34, 0.062, 0.055, 0.037 and

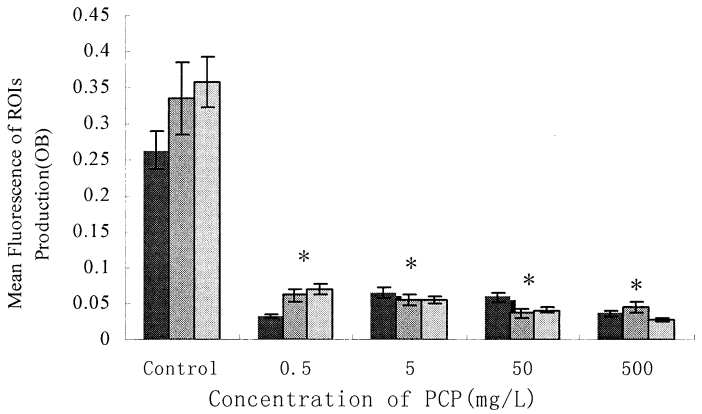

Figure 3. Change of in vitro ROIs production fluorescence in the crucian carp macrophages.

0.045, respectively ($P<0.05$); mean RLU of 3 min were 0.36, 0.070, 0.055, 0.041 and 0.028 respectively ($P<0.05$). Compared to the control group, the four experimental groups all showed significant statistical differences, which indicated that ROIs production was greatly suppressed by PCP. There was no obvious dose-dependent relationship found, but a time-dependent relationship could be observed. The RLU of the control group and 0.5mg/L group increased with time, in contrast the 5, 50 and 500 mg/L groups did not show the same pattern.

Exposure to PCP significantly suppressed phagocytosis in the in vitro test(Table 1), but had no obvious impact on phagocytosis in the in vivo test. In the in vitro experiment, mean fluorescence of the 0.5, 5, and 50 mg/L exposure groups were 82.7 ($P<0.05$) 77.6 ($P<0.05$) and 62.2 ($P<0.05$), respectively; with all showing significant suppression compared with the control group (114.5). The suppression was greatest at the 500 mg/L, as no obvious fluorescence could be detected. However, in the in vivo experiment, mean fluorescence of the low (0.027 mg/L), medium (0.053 mg/L) and high (0.13 mg/L) concentration groups were 72.5 ($P>0.05$), 54.1 ($P>0.05$) and 56.4 ($P>0.05$), respectively. No significant changes could be seen compared to the control (63.0).

Table 1. Change of in vitro and in vivo phagocytosis in macrophages

in vitro test	PCP (mg/L)			
	Control	0.5	50	500
Mean fluorescence	114.5 ± 2.2	72.5± 4.0	77.6 ± 4.4*	62.2 ± 5.7*
in vivo test	PCP (mg/L)			
	Control	0.027	0.053	0.13
Mean fluorescence	63.0 ± 3.2	82.7 ± 4.2	54.1 ± 5.4	56.4 ± 5.0

Values are means±S.D., * $P<0.05$ vs Control

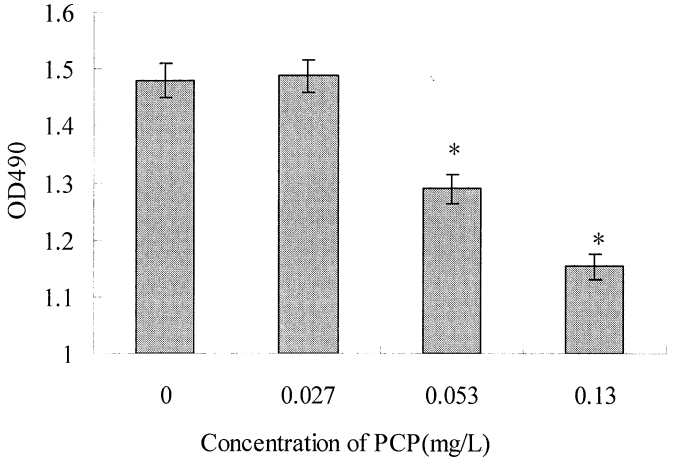

Figure 4. Change of IgM level in the crucian carp serum.

Following exposure to PCP in vivo, mean OD of the control, low, medium and high concentration groups were 1.48, 1.49, 1.29, and 1.15, respectively. It indicated that IgM levels were significantly decreased with the medium and high concentrations. However, IgM levels didn't change significantly with the low concentration group.

The immunotoxicity of PCP to cellular and humoral immunity of the crucian carp via both in vitro and in vivo tests was determined in the study. In vitro tests showed that PCP has great suppression on viability of pronephric macrophages, production of ROIs and phagocytosis. Purschke et al. (2002) reported that tetrachlorohydroquinone (TCHQ), one metabolite of PCP, has a suppressive effect on viability of human macrophages. Anderson and Anderson (2000) also reported that chlorothalonil could suppress production of ROIs in *Morone saxatilus*. These data are consistent with the results of our study. PCP lowers cell viability mainly through dissociating substrates, which are used for ATP synthesis. In the process of macrophage respiration burst, the stimulator PMA can induce NADPH oxidase activity on the cell membrane, thus helping the oxygen molecule to acquire one electron and become the superoxide anion. Pinelli et al. (1994) had revealed that lindane affected cell NADPH oxidase activity, so we hypothesize that suppression of ROIs is through the same pathway.

In the in vitro test, phagocytosis was decreased significantly. Our results support the result of Fournier et al. (1998). No significant impact of PCP to macrophage phagocytosis was detected in our in vivo test, but a decreasing tendency could still be seen. This may suggest that PCP does exert impact on macrophages in fish, but the effect might be weakened by some underlying compensating mechanisms, such as nitric oxide production (Roszell and Anderson 1996) and cytokines etc.

Serum IgM levels decreased significantly after fish were exposed to PCP. IgM is produced by B cells, T helper cells also affect the production of IgM, and there are estrogen receptors on their surfaces. Because PCP has an estrogen-like function in fish (Flouriot et al. 1995), it is likely that PCP binds to surface receptors of B cells or T helper cells so as to suppress their functions and reduce level of IgM production.

Our study reveals that PCP, one of the EEDs, has fairly high immunotoxicity to crucian carp. Previous research has shown that EEDs can affect the immune system in mammals and ocean fish. On one hand, EEDs can change intrinsic immune response and cause immunotoxicity directly. On the other hand, EEDs can also affect the immune system by changing the endocrine system, and vice versa, because there are broad interrelationships between the immune and endocrine systems. In addition, the fish specific immune system is the probable target of EED function. It remains necessary to further examine the immunotoxic mechanisms of PCP.

Acknowledgment. This study is supported by the *National Natural Science Foundation of China* (Grant No. 30070089), the *High-Tech Development Plan* (Grant No. 2001AA640601-4) and the 985 Program of China.

REFERENCES

U.S. Department Of Health And Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry (2001) Toxicological profile for pentachlrophenol: Health effects chapter. PB/2001/109106/AS Atlanta, GA, USA

Anderson C B, Anderson R S (2000) Suppression of superoxide production by chlorothalonil in striped bass (*Morone saxatilus*) macrophages: The role of cellular sulfhydryls and oxidative stress. Aquat Toxicol 50: 85–96

Blakley B R, Yole M J, Brousseau P, Boermans H, Fournier M (1998) Effect of pentachlorophenol on immune function. Toxicology 125: 141–148

Chen Y, Wang S, Han X, Hou Y (2003) Purification and partial characterization of immunoglobulin M from *Carassius auratus* serum and skin mucus. Zool Res 24:111-115

Flouriot G, Pakdel F, Ducouret B, Valotaire Y (1995) Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. J Molec Endocrinol 15:143-51

Food and Agriculture Organization of the United Nations, United Nations Environment Programme (1991; amended 1996) Operation of the prior informed consent procedure for banned or severely restricted chemicals in international trade: decision guidance documents-Pentachlorophenol,

Chapter Pesticides set 4, FAO, Rome, Italy
Fournier M, Lacroix A, Voccia I, Brousseau P (1998) Phagocytic and metabolic activities of macrophages from mummichog naturally exposed to pulp mill effluents in the Miramichi River. Ecotoxicol Environ Saf 40: 177-183
Karrow N A, Boermans H J, Dixon D G, Solomon K R, Whyte J J (1999) Characterizing the immunotoxicity of creosote to rainbow trout (*Onchorhynchus mykiss*): a microcosm study. Aquat Toxicol 45: 223–239
Martens D, Zhang A, Chen J, Schramm K W, Kettrup A (2000) Monitoring of chlorinated and other organic trace contaminants in selected stretches of the Liao and Yangtse River (China). Joint Research Center, EUR 19531 CN European Communities: 70
Piemonti L, Monti P, Zerbi A, Balzano G, Allavena P, Di Carlo V (2000) Generation and functional characterization of dendritic cells from patients with pancreatic carcinoma with special regard to clinical applicability. Cancer Immunol Immunother 49: 544-550
Pinelli E, Cambon C, Tronchere H, Chap H, Teissie J, Pipy B (1994) Ca^{2+}-dependent activation of phospholipases C and D from mouse peritoneal macrophages by a selective trigger of Ca^{2+} influx, γ-hexachlorocyclohexane. Biochem Biophys Res Commun 199: 699-705
Purschke M, Jacobi H, Witte I (2002) Differences in genotoxicity of H_2O_2 and tetrachlorohydroquinone in human fibroblasts. Mutat Res Genet Toxicol Environ Mutagen 513: 159-167
Roszell L E, Anderson R S (1996) Effect of chronic in vivo exposure to pentachlorophenol on non-specific immune functions in *Fundulus heteroclitus*. Mar Environ Res 42: 191-194
Roszell L E, Anderson R S (1997) Hydrogen perroxide production and bactericidal activity in pronephros macrophage sub-populations from *Fundulus heteroclitus* following exposure to pentachlorophenol. Mar Environ Res 43: 1-9
Zhu L, Li Y, Ci Y (1999) The studies on the respiratory burst changes with age in rabbit peritoneal macrophages with chemiluminescence method. Acta Scien Natural Univer Pekinen 35: 172-177