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Pentachlorophenol impact assessment of haematological parameters in olive flounder, *Paralichthys olivaceus*

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The toxic effects of pentachlorophenol (PCP) on haematological parameters in olive flounder (*Paralichthys olivaceus*) after chronic exposure to dietary PCP (0.1, 0.5 and 1.0 mg/kg diet) for 2, 4 and 6 weeks were studied. A significant decrease in total RBC count, haemoglobin concentration and hematocrit value was noted in fish exposed to PCP compared to the non-exposed fish. The PCP treatment group showed significantly lower concentration of serum total protein and albumin, and significantly higher serum chloride, magnesium and total bilirubin levels compared with those in the control group. However, PCP had no major effects on serum glucose, total cholesterol, phosphate and calcium ions in flounder. These results demonstrated that PCP have induced adverse haematological impacts in the olive flounder, *Paralichthys olivaceus*. Because we found damages in blood-forming function and disruption in blood homeostasis due to chronic exposure to PCP, it is needed to develop further experimental studies for the risk assessment of this environmental pollutant.

Key words : Pentachlorophenol, Haematological parameter, Impact assessment, *Paralichthys olivaceus*

Introduction

Pentachlorophenol (PCP) has been used as fungicide, molluscide, insecticide and herbicide and as preservatives (Seiler, 1991; Shen *et al.*, 2005). The relatively high volatility of PCP and water solubility of its ionized form have led to widespread contamination of the environment with this compound (Crosby *et al.*, 1981). Due to its biocidal properties, PCP negatively affects non-target organisms in soil and water at relatively low concentrations. Most aquatic invertebrates and vertebrates are affected by PCP at concentrations below 1 mg/liter in acute exposure (Davis and Hoos, 1975; Borthwick and Schimmel, 1978). The US Environmental Protection Agency (US EPA) has listed PCP as a priority pollutant because of its proven carcinogenicity and toxicity, as well as of the large number of known PCP-contaminated sites worldwide (US EPA, 1988).

In short-term studies, the LC_{50} values for PCP are generally less than 1 mg/L, and, in many cases, even less than 0.1 mg/L (Inglis and Davis, 1972; Davis and Hoos, 1975). PCP toxicity on aquatic organisms can be influenced by temperature (Rusink and Smith, 1975; Crandall and Goodnight, 1959), pH (Saarikoski and Viluksela, 1981) and dissolved oxygen (Gupta *et al.*, 1983). Even though it is known that fish exposed to PCP exhibit immunotoxicity and deterioration in reproduction and development (Chen *et al.*, 2004), little has been done to determine which physiological system of fish are

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the most sensitive indicators of PCP contamination.

Measurement of physiological parameters is a commonly used diagnostic tool in aquatic toxicology and biomonitoring (McDonald and Milligan, 1992; Folmar, 1993; Soimasuo *et al.*, 1995; Kang *et al.*, 2003; Jee *et al.*, 2004). In addition, various haematological factors have been shown to be sensitive indicators of changes in ecological condition (Payne *et al.*, 1978; Bansal *et al.*, 1979). Therefore, blood parameters are increasingly being used as indicators of the physiological or sublethal stress responses in fish.

The aim of this study was to evaluate the effects of PCP on the haematological parameters in olive flounder, *Paralichthys olivaceus* after chronic dietary PCP exposure.

Materials and Methods

Experimental animals

Cultured olive flounder were obtained from a local fish farm in Pohang, Kyongbook, Korea. Prior to exposure, fish were held for three weeks for acclimatization and evaluation of fish health under laboratory conditions in a 12:12 h light/dark cycle. During acclimatization fish were fed a basal diet (Table 1) twice daily (2% body weight for each meal at approximately 10:30 and 16:30 h). After acclimatization, fish (mean body weight 49.7 ± 3.9g) were selected for the chronic exposure experiments.

Diet preparation

Ingredients of basal diet used in this study are

Table 1. Composition of the basal diet

Ingredients	g/Kg
Casein, vitamin free	335
Gelatin	75
Corn starch	280
Dextrin	140
Squid liver oil	50
Soy bean oil	30
Carboxymethylcelulose	30
Protease	5
Cellulose	15
Vitamin premix[1]	10
Mineral premix[2]	30

[1]The vitamin mix provided the following in mg/Kg diet: vitamin A (500,000 IU/g), 8; vitamin D_3(1,000,000 IU/g), 2; vitamin K, 10; vitamin E, 100; thiamine, 10; riboflavin, 20; pyridoxine, 20;vitamin C, 50; nicotinic acid, 150; folic acid, 10; vitamin B_{12}, 0.02; biotin, 2; inositol, 400; choline chloride, 2,000; panthothenate, 200.

[2]Contains (as g/Kg premix): NaCl, 43.3; $MgSO_4 \cdot H_2O$, 136.6; $NaH_2PO_4 \cdot 2H_2O$, 86.9; KH_2PO_4, 239.0; $Ca(H_2PO_4)_2 \cdot H_2O$, 135.3; $ZnSO_4 \cdot 7H_2O$, 21.9; Fe-citrate, 29.6; Ca-lactate, 303.89; $AlCl_3 \cdot 6H_2O$, 0.15; KiO_3, 0.15; Na_2SeO_3, 0.01; $CuCl_2$, 0.2; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

presented in Table 1. The experimental diet was supplemented with PCP at levels of 0, 0.1, 0.5 and 1.0 mg/kg diets. Ingredients of each diet had been thoroughly mixed in a Hobart mixer. Thereafter, PCP dissolved in ethyl alcohol at required amount (Sigma, St. Louis, MO) was added and the whole mixture was blended. The same amount of alcohol was added to the control diet. The moist mixture was extruded through a 3 mm diameter module. The resulting moist pellets were air-dried at room temperature to moisture content of about 10%. Pellets were ground into small pieces, sieved to obtain appropriate sizes and stored frozen in plastic bags at -20 ℃ until fed.

Experimental Design and Exposure Regime

Olive flounder (*Paralichthys olivaceus*) were placed in triplicate 150 L aquaria with continuous supply of sea water containing air stones to maintain dissolved oxygen levels greater than 75% saturation for the whole exposure period. After acclimatization the fish were distributed in test chambers, each housing ten fish. The test containers were supplied with continuous flow-through water (flow = 4 L/min). Ten fish per exposure concentration were captured for analysis and anesthetized with buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO) after the 2nd, 4th and 6th week of exposure. They were starved for 48 h before blood sampling.

Blood sample and haematological assay

Blood samples were taken from each fish by puncture of the caudal vessel using heparinized syringes. For serum collection blood was collected without heparin. Blood was allowed to coagulate at room temperature for 2 h and serum was obtained by centrifugation, i.e., approximately 1.5 ml, at 3,000 X g for 12 min at 4 ℃ (MIKRO 22R, Hettich, Germany) and then stored at -80 ℃ until analyzed. Hematocrit (Ht) was determined by microhematocrit technique using capillary tubes by centrifugation at 12,000 rpm for 5 min. Hemoglobin (Hb) was determined spectrophotometrically (540 nm) using cyanomethemoglobin method. Hb concentration was expressed as g/dL. Red blood cell (RBC) counts were estimated using a Neubauer hemocytometer (Hesser, 1960). Hematological analyses were done by techniques described by Tvedten (1989) and Campbell and Murru (1990).

Serum chemistry

Serum samples were analyzed for calcium (Sigma Diagnostics kit 588, colorimetric method), magnesium (Sigma Diagnostics kit 595, colorimetric method), phosphorus (Sigma Diagnostics kit 360, colorimetric method) and chloride (Sigma Diagnostics kit 461, colorimetric method). Blood albumin (BCG method), total protein (colorimetric method), glucose (glucose oxidase/peroxidase method), total bilirubin (Michaelsson method) and total cholesterol (colorimetric method) were determined on serum using Sigma Diagnostic Kits.

Statistical analysis

Statistical analysis was performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, USA). Significant differences between groups were determined using one-way ANOVA and Duncan's test for multiple comparisons (Duncan, 1955). The level of significance was established at $P<0.05$.

Results

Hematological property

Data for hematological property of olive flounder are presented in Table 2. Blood parameters including red blood cell (RBC) count, hemoglobin (Hb)

Table 2. Haematological properties in olive flounder, *Paralichthys olivaceus* exposed to dietary pentachlorophenol for 6 weeks

Parameter	Time (weeks)	Group			
		Control	0.1 mg/Kg	0.5 mg/Kg	1.0 mg/Kg
RBC count ($X10^4/mm^3$)	2	307.8 ± 12.6^a	249.9 ± 5.3^b	246.4 ± 7.7^b	218.3 ± 5.7^c
	4	293.1 ± 8.5^a	244.8 ± 16.5^{bc}	254.7 ± 10.4^b	213.8 ± 3.3^c
	6	313.9 ± 8.1^a	273.7 ± 3.4^b	256.3 ± 10.3^b	247.1 ± 17.1^b
Ht (%)	2	30.6 ± 1.0^a	25.9 ± 0.7^b	26.3 ± 1.4^b	24.1 ± 0.8^b
	4	29.0 ± 1.5^a	24.5 ± 1.2^b	23.0 ± 1.7^b	22.8 ± 1.1^b
	6	28.9 ± 0.6^a	25.0 ± 0.6^b	22.3 ± 1.0^b	22.1 ± 1.4^b
Hb (g/dL)	2	5.8 ± 0.2^a	$5.1 \pm 0.1a^{bc}$	5.3 ± 0.3^{ab}	4.6 ± 0.2^c
	4	5.3 ± 0.3^a	4.5 ± 0.3^b	4.2 ± 0.2^b	4.4 ± 0.2^b
	6	5.1 ± 0.1^a	4.7 ± 0.3^{ab}	4.7 ± 0.2^{ab}	4.2 ± 0.2^b
MCH (pg)	2	18.9 ± 0.6	20.3 ± 0.6	21.7 ± 0.8	20.9 ± 0.9
	4	18.3 ± 1.0	18.4 ± 1.0	16.5 ± 1.2	20.4 ± 1.1
	6	16.4 ± 0.5	17.1 ± 1.3	18.3 ± 1.0	17.1 ± 0.9
MCHC (%)	2	18.9 ± 0.5	19.5 ± 0.4	20.5 ± 1.0	18.9 ± 0.5
	4	18.6 ± 1.0	18.3 ± 1.0	18.4 ± 1.0	19.1 ± 0.7
	6	17.8 ± 0.4	18.8 ± 1.2	21.0 ± 1.4	19.2 ± 1.5
MCV (μm^3)	2	100.2 ± 4.0	103.7 ± 2.0	106.7 ± 4.6	110.4 ± 2.5
	4	99.2 ± 4.8	100.8 ± 2.3	91.2 ± 7.6	106.8 ± 4.3
	6	92.2 ± 1.3	91.4 ± 2.2	87.2 ± 1.5	91.9 ± 7.7

All data are presented as means ± SE (n=10). Means in each row with a different superscript are significantly different ($P<0.05$). RBC: red blood cell, Ht: hematocrit, Hb: hemoglobin, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume.

concentration, hematocrit (Ht) levels decreased with an increase in exposure time of pentachlorophenol (PCP) to the fish. The most predominant haematological changes was a significant decrease of the RBC count in fish exposed to 0.5 and 1.0 mg/Kg PCP-treated fish. Hb concentration and Ht were also significantly decreased in flounders treated with concentrations higher than 0.1 mg/Kg PCP ($P<0.05$).

Serum inorganic chemistry and osmolality

Serum inorganic ion levels are shown in Table 3. A significant decrease ($P<0.01$) in Cl ion was observed in the 1.0 mg/Kg diet group at both 4th and 6th week examined. Serum magnesium concentrations were increased in flounder exposed to 0.5 and 1.0 mg/Kg diet groups at 4th week ($P<0.05$). Phosphate and calcium indices revealed a marginal or no differences from control values.

Table 3. Serum inorganic parameters in olive flounder, *Paralichthys olivaceus* exposed to dietary pentachlorophenol for 6 weeks

Parameter	Time (weeks)	Group			
		Control	0.1 mg/Kg	0.5 mg/Kg	1.0 mg/Kg
Chloride (mM)	2	147.2±4.1	149.6±4.6	145.3±4.0	154.5±4.1
	4	146.8±3.2^{a}	140.3±6.3^{ab}	154.4±6.0^{ab}	157.3±5.9^{b}
	6	149.4±3.0^{a}	140.5±6.4^{a}	146.0±3.1^{a}	160.7±2.1^{b}
Phosphate (mM)	2	2.40±0.02	2.38±0.04	2.49±0.06	2.42±0.05
	4	2.51±0.06	2.40±0.09	2.32±0.04	2.48±0.08
	6	2.48±0.05^{a}	2.27±0.02^{b}	2.51±0.06^{a}	2.46±0.07^{a}
Magnesium (mM)	2	1.33±0.02^{a}	1.34±0.03^{a}	1.42±0.02^{b}	1.39±0.02^{ab}
	4	1.37±0.03^{ab}	1.33±0.05^{a}	1.49±0.05^{b}	1.50±0.09^{b}
	6	1.39±0.04	1.40±0.03	1.54±0.05	1.44±0.07
Calcium (mM)	2	2.80±0.04	2.84±0.04	2.77±0.04	2.57±0.26
	4	2.46±0.24	2.79±0.04	2.80±0.05	2.77±0.03
	6	2.77±0.03	2.80±0.07	2.82±0.04	2.70±0.07

All data are presented as means±SE (n=10). Means in each row with a different superscript are significantly different ($P<0.05$).

Table 4. Serum organic parameters in olive flounder, *Paralichthys olivaceus* exposed to dietary pentachlorophenol for 6 weeks

Parameter	Time (weeks)	Group			
		Control	0.1 mg/Kg	0.5 mg/Kg	1.0 mg/Kg
Total protein (g/dL)	2	3.95±0.07	3.99±0.09	3.94±0.12	3.70±0.12
	4	3.81±0.10^{ab}	3.87±0.18^{a}	3.77±0.23^{ab}	3.35±0.15^{a}
	6	4.15±0.10^{a}	3.80±0.17^{ab}	3.72±0.10^{b}	3.54±0.11^{b}
Albumin (g/dL)	2	1.00±0.02	1.00±0.02	0.99±0.03	0.93±0.03
	4	0.95±0.03	0.97±0.05	0.95±0.06	0.89±0.03
	6	1.04±0.03^{a}	0.95±0.04^{b}	0.95±0.02^{b}	0.88±0.02^{b}
Glucose (mg/dL)	2	39.2±2.9^{a}	36.2±3.9^{a}	41.4±2.8^{a}	56.9±4.3^{b}
	4	46.3±3.0	45.5±5.6	49.8±7.9	45.7±4.2
	6	49.0±4.3	51.0±4.8	45.6±3.2	54.1±5.4
Total bilirubin (mg/dL)	2	0.38±0.03	0.35±0.03	0.34±0.03	0.47±0.06
	4	0.33±0.02^{a}	0.35±0.04^{a}	0.53±0.09^{b}	0.63±0.06^{b}
	6	0.36±0.02^{a}	0.39±0.04^{a}	0.49±0.06^{a}	0.65±0.07^{b}
Total cholesterol (mg/dL)	2	252.5±9.2	243.9±7.6	229.2±12.8	238.7±9.6
	4	257.0±6.9	248.5±12.2	246.6±11.8	249.5±9.6
	6	251.6±6.1	254.5±15.4	239.0±8.9	243.0±10.6

All data are presented as means±SE (n=10). Means in each row with a different superscript are significantly different ($P<0.05$).

Serum organic chemistry

The changes of serum organic compositions in fish exposed to PCP are presented in Table 4. Serum total protein and albumin concentrations were decreased in flounder exposed to 0.5 and 1.0 mg/Kg diet groups at 6th week. Serum glucose concentrations showed a significant increase after 2 weeks at the highest concentration (1.0 mg/Kg) group ($P<0.05$), but the increase was not maintained thereafter. Serum total bilirubin levels were increased by 60.6 and 91.0% in flounder exposed to 0.5 and 1.0 mg/Kg for 4 weeks, respectively ($P<0.05$). Plasma total cholesterol concentration in PCP exposed fish was slightly lower than control fish, but no significant differences were observed among experimental groups.

Discussion

Pentachlorophenol (PCP) are persistent organic pollutants in the environment. As anaerobic biodegradation of PCP is relatively slow, they are preserved in sediments for a very long time. The highest concentration of phenanthrene and PCP in aquatic environments usually has been found in aquatic sediments (Maatela *et al.*, 1990, Abrahamsson and Klick, 1991). Therefore, the toxicity of PCP to aquatic organisms will remain for a very long time. Generally, lipophilic materials are taken up in fish partly by the gills, and these substances are expected to accumulate in the cell membranes where they may affect cell membrane permeability and membrane-located transport mechanisms (Payne *et al.* 1978). PCP had bioaccumulation potential in different trophic organisms once it entered the aquatic environment and would finally reach human beings through foodstuffs (Eduljee, 1999).

Pleuronectid flatfishes are recognized as being well suited for biomonitoring in the field and laboratory (Eggens *et al.*, 1996; Hylland *et al.*, 1996; Jee *et al.*, 2004). Benthic fish should be a suitable biomarker to monitor sediment pollution since they are in contact with contaminated sediments and more likely to suffer adverse effects to their osmoregulatory system than pelagic species. Therefore flounder is an excellent sentinel species for environmental monitoring.

Exposure to PCP in the present investigation showed remarkable haematological stress at high concentrations. The observation of decrease in RBC count, hemoglobin concentration and hematocrit level in this study may indicate the disruptive action of PCP. Our studies provide evidence that PCP causes erythrocyte hemolysis. Decline in RBC count, hemoglobin concentrations and hematocrit presumably reflect erythrocyte hemolysis. The decrease in hemoglobin concentration may be due to either an increase in the rate at which hemoglobin is destroyed or a decrease in the rate of hemoglobin synthesis, and may be due to the disruptive action of the PCP on the erythropoietic tissue as a result of which the viability of the cells might be affected.

Fish under stress also mobilize protein to meet energy requirements needed to sustain increased physical activity (Sievers *et al.*, 1995). Decreased serum total protein and albumin levels in the present study after six weeks exposure may be attributed to stress-mediated mobilization of these compounds to fulfill an increased demand for energy by the fish to cope with detrimental conditions imposed by the toxicant. Bilirubin is a common bile pigment formed in the liver from the breakdown of heme and other porphyrin rings (Kaplan and Szabo, 1979). Elevated level of serum bilirubin in the present study of PCP-exposed olive flounder may be due to severe cellular damage leading to impaired cellular function. Hyperbilirubinaemia suggests

liver damage or obstruction of the bile ducts. In some forms of anaemia, particularly of haemolytic type, the level may rise due to the inability of the liver to pass the increased quantity of the pigment. Thus the elevated level of serum bilirubin suggests liver damage(Jyothi and Narayan, 1999).

In conclusion, our results show that PCP might have induced haematological impact in the olive flounder, *Paralichthys olivaceus*, this reflect that some of the damages in blood-forming function and disruption of blood homeostasis of olive flounder was due to chronic exposure to PCP.

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