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Effects of Long-Term Exposure of Low-Level Diesel Oil on the Antioxidant Defense System of Fish

J. F. Zhang, H. Shen, T. L. Xu, X. R. Wang, W. M. Li, Y. F. Gu

State Key Laboratory of Pollution Control and Resources Reuse, School of Environment, Nanjing University, Nanjing, 210093, People's Republic of China

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Reactions generating free radicals in biological systems, especially oxygen free radicals, are complex and often involve chain mechanisms resulting in various kinds of radicals. Aerobic organisms contain a variety of defenses to protect against the adventitious production of oxygen radicals that may arise as a result of uncoupling at various electron transfer sites or via autooxidation reactions. As the important reactive oxygen removal system in the body of aerobic organisms, the antioxidant defense system plays a crucial role in maintaining cell homeostasis. Its induction reflects a specific response to pollutants (Doyotte et al. 1997) and has been proposed as a biomarker of contaminant-mediated oxidative stress in a variety of marine organisms (Livingstone et al. 1992; Gamble et al. 1995; Regoli et al. 1998). On normal physiological conditions, the reactive oxygen generated from metabolism of extraneous chemicals in the body can be removed well by the antioxidant defense system. But when living organisms are exposed to redox-cycling compounds, if the generation rate of reactive oxygen is beyond the removal capacity of the antioxidant defense system, oxidative stress occurs and, as a result, some oxidative damage may appear, for example, lipid peroxidation, enzyme inactivation, DNA strand breaks, covalent binding to protein and nucleic acid, and so on.

In recent years, oil pollution has been a global environmental issue, in that it has threatened the oceanic and inland aquatic breeding ecosystems. Evaluation and prediction of the effects of oil pollution on the water environment has become an urgent and important job. Sublethal effects of low-concentration contaminants on organisms, which may be difficult to detect and assess, have been reported to result from chronic exposure to petrochemicals (Engelhardt, 1983; IARC 1989; McBee and Bickham 1990; Khan and Ryan 1991; Stubblefield et al. 1995 a, b). Such damage was observed after long-term exposure instead of a short period. So the effects of long-term exposure of low-concentration pollutants on the ecosystem are of concern. At home or abroad, studies on the toxicological effects of oil on physiological and biological processes in aquatic organisms have been carried out for a long time. However, few reports have been published concerning the effects of long-time static exposure of low-concentration oil on the antioxidant defenses of fish. In this experiment, the larvae of goldfish *Carassius auratus*, one of the main economical fish species of Taihu Lake, were chosen as the model organism. The objective was to study the effects of long-term exposure of low-concentration no. 20 diesel oil on the antioxidant defense system of larval goldfish, and if possible, provide some clues for further studies, such as development of biochemical indices as early warning biomarkers of low-level oil exposure in the aquatic environment.

Correspondence to: X. R. Wang

MATERIALS AND METHODS

In this research, experimental reagents were of analytical or ultra-pure grade from home or abroad (Sigma). The soluble fraction of no. 20 diesel oil was the contaminant at six experimental concentrations (0.005, 0.01, 0.05, 0.1, 0.5, 1.0 mg/L), with 40 days of exposure. The oil stock solution was prepared as follows, according to Rayburn et al. (1996). No. 20 diesel oil was added to distilled water, and then the solution was mixed slowly for 18 hr continuously and allowed to stand an additional 5 hr for the oil droplets to rise to the surface. Then the oil solution below the surface layer was taken out by siphoning and served as the stock solution. An accurate concentration was measured by an RF-5000 fluoro-spectrophotometer, with fluorescence at 332 nm determined, with activation at 231 nm. The stock solution was held at 4℃ in a refrigerator and the container remained capped during the entire experimental period to minimize evaporation.

Goldfish (*Carassius auratus)* were captured from a local aquatic breeding base, with average body length and weight about 11.3 ± 0.67 cm and 22.1 ± 0.46 g, respectively. Before exposure the fish were acclimated to the water, which had been dechlorinated by active carbon for 10 days, with the total mortality of fish near zero. Air flow was continuous and artificial dry food was provided once a day.

The fish were divided into seven groups (7 fish each), and each group corresponded to an oil-exposed concentration, while the unexposed group served as control samples. These fish were randomly selected into the aquaria with the rate of fish/water 3.37 g/L. During the experiment, water pH was 7.0 ± 0.1 and water temperature was 25 ± 2℃, with hardness about 100 mg/L as $CaCO_3$.

After a 40-d exposure, seven goldfish were taken from each group for parallel samples. Goldfish samples were weighed, dissected and their livers were separated for weight determination after rinsing in physiological salt water. About 0.30 g of liver tissue was homogenized after addition of 3.0 mL of 10.0 mM Tris buffer (pH 7.5) for detection of enzyme activities. About 0.10 g of liver tissue was homogenized after addition of 1.0 mL of 1.0mmol/L EDTA and 10 μL $HClO_4$ for measurement of GSH (reduced glutathione). The extracts were centrifuged at 1×10^4 rpm for 10 minutes at 4℃ and preserved at -85℃ for analysis.

Determination of protein in fish livers was carried out by the method of Bradford (1976). In brief, protein reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol and adding 100 mL 85% (w/v) phosphoric acid to this solution. The absorbance of 0.1 mL protein solution mixed with 5.0 mL protein reagent was measured by a UV-220 spectrophotometer at 595 nm after 2 min. The content of protein was calculated by the corresponding absorbance according to a standard curve. Bovine serum albumen (BSA, Sigma) was used as the protein standard.

Catalase activity was assayed by ultraviolet spectrophotometry (Xu et al. 1997). A 10 μL sample was added to 3.0 mL of H_2O_2 phosphate buffer pH 7.0 (0.16 mL of 30% H_2O_2 to 100 mL of 0.067 M phosphate buffer) and the variation of H_2O_2 absorbance in 60 seconds was measured with a UV-220 spectrophotometer at 250 nm. One unit of enzyme activity is defined as the amount of the enzyme which decreased the concentration of H_2O_2 by 50% in 100 seconds at 25℃.

Liver SOD (superoxide dismutase) activity was determined by measuring the inhibition of the auto-oxidant of pyrogallol using a modification of method of Marklund and Marklund (1974). Samples were assayed in a solution of 8.7 mL of

50 mM phosphate buffer pH 8.24 and 0.3 mL of 3 mM pyrogallol (dissolved in 10 mM HCL). The rate of pyrogallol auto-oxidation was measured with a UV-220 spectrophotometer at 325 nm. One unit of SOD activity is defined as the amount of the enzyme which gave 50% inhibition of the oxidation rate of 0.1 mM pyrogallol in one ml of solution at 25℃.

Determination of GSH was performed by the method of Hissin and Hilf (1976). To 0.5 mL of supernatant, 4.5 mL of phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 mL) contained 100 μL of the diluted tissue supernatant, 1.8 mL of phosphate-EDTA buffer, and 100 μL the OPT (O-Phthalaldehyde) solution, containing 100 μg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with activation at 350 nm.

Se-GPx (Se-dependent glutathione peroxidase) activity was measured according to Hafeman et al. (1973) with a slight modification. The reaction mixture contained GSH, NaN_3-PBS pH=6.5, H_2O_2, TCA (trichloroacetic acid), Na_2HPO_4, DTNB (5.5'-Dithiobis(2-nitrobenzoic acid)). The absorbance at 423 nm was monitored on a UV-220 spectrophotometer at 37℃. One unit of enzyme activity is defined as the amount of enzyme that oxidised 1 μmol/L of GSH per min per mg of protein, while the specific measurement of non-Se-GPx activity was allowed for.

GST (glutathione S-transferase) activity was measured according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Assays were performed in a reaction mixture containing 1.05 mL of 100 mM Tris buffer (pH 7.4), 50 μL CDNB (1 mM), 50 μL GSH (1 mM) and 50 μL tissue homogenate. Before use, the GSH required for the assay was dissolved in Tris buffer. The CDNB was dissolved in ethanol. In all cases the final concentration of ethanol in the assay mixture did not exceed 5% (v/v). Blanks were achieved in the same conditions, but replacing the sample with Tris buffer. Enzyme activity was determined by monitoring changes in absorbance at 340nm (which translates the rate of CDNB conjugation with GSH (E_{340}CDNB-GSH conjugation = 9.6 $mM^{-1}cm^{-1}$)) for 2 min at constant temperature with a UV-220 spectrophotometer. This GST activity is expressed as nmoles/min/mg protein.

Experimental data were expressed as mean±SD and analyzed using one-way ANOVA. Significant differences were defined to exist between two groups as P is less than or equal to 0.05.

RESULTS AND DISCUSSION

After a 40-d oil exposure, changes in catalase activity in the livers of *Carassius auratus* are shown in Figure 1. The enzyme activity in Group II was not significantly different from that in the control group, which demonstrated that such a low concentration as 0.005 mg/L can hardly activate catalase. In contrast, catalase activity in Group III, which was 2.9 times that in the control group, was induced significantly by oil pollution, while the catalase activities in other groups didn't show a significant difference from control organisms. This indicated, when oil level reached 0.01 mg/L, catalase activity was increased firstly and then decreased rapidly when oil level was over 0.01 mg/L, which should be the result of the increase in Se-GPx activity (shown in Figure 5).

Changes in SOD activity in livers of exposed *Carassius auratus* are shown in Figure 2. It was observed that SOD activity was induced significantly by oil pollution in Group II and III ($p<0.01$), with values 1.54 and 1.58 times the control

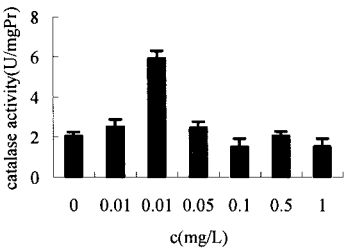

Figure 1. Effects of oil pollution on catalase activity in livers of *Carassius auratus*

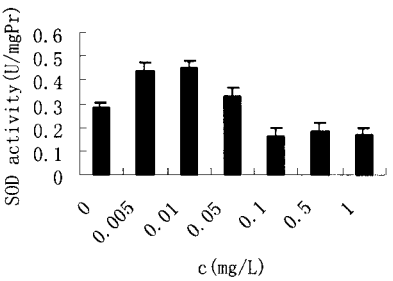

Figure 2. Effects of oil pollution on SOD activity in livers of *Carassius auratus*

group, respectively. With oil concentration increasing, SOD activity was inhibited gradually in the next groups. As a result of slight oxidative stress, SOD activity made a counteractive response at low-intermediate doses, and the following induction might suggest a higher oxyradical formation. 0.1 mg/L can be suggested as the threshold value for response of larval fish to oil pollution.

Changes in GSH level in livers of *Carassius auratus* are shown in Figure 3. The analytical results indicated that a concentration as low as 0.005 mg/L was not enough to affect GSH level. From Group III on, oil pollution increased GSH levels significantly ($p<0.01$). GSH levels in Group III and IV were 1.71 and 1.86 times that in the control group, respectively. With oil concentration increasing, GSH levels of the remaining three groups showed no significant differences compared to the control group. GSH is often the first line of defense against oxidative stress. GSH levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis; however, a severe oxidative stress may suppress GSH levels due to the loss of adaptive mechanisms and the oxidation of GSH to its oxidized form, GSSG (Chen and Lin 1977). In this experiment, oil exposure didn't lead to GSH depletion, which might be the result of correction by the synthesis of new glutathione molecules and the reduction of GSSG catalysed by GRd.

Changes in Se-GPx activity in livers of *Carassius auratus* after exposure are shown in Figure 4. Oil pollution induced Se-GPx activity significantly in each exposure group ($p<0.05$). The enzyme activities in exposure groups were, respectively, 1.51, 2.26, 1.71, 1.35, 1.33, and 1.27 times that in the control group. Se-GPx carries out its detoxicative function as the catalyst of substrate GSH, so the induction might reflect increased peroxidation as a result of oil exposure. As shown in Figure 3, although GSH levels took on a descending trend, it was still higher than that in control group. It was seen from figures 1 and 2 that at higher concentrations the catalase and SOD activities were already lower than those in the control group. Thus, it could be concluded, with increasing oil concentration, GSH and GPx played dominant roles in the process of gradual detoxication.

Changes in GST activity in livers of *Carassius auratus* are shown in Figure 5. In general, oil pollution assumed an inhibitive function on GST activity. The enzyme activity in Group II was not significantly different from that of the control group. GST activities among the other five groups were not significantly different from each other, while their values were 0.73, 0.76, 0.67, 0.7, and 0.72 times that in the control group, respectively.

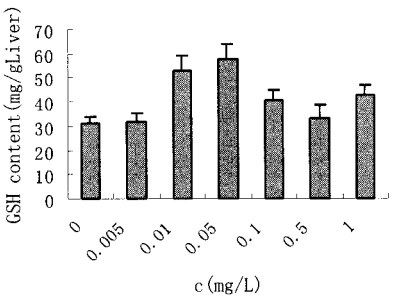

Figure 3. Effects of oil pollution on GSH content in livers of *Carassius auratus*

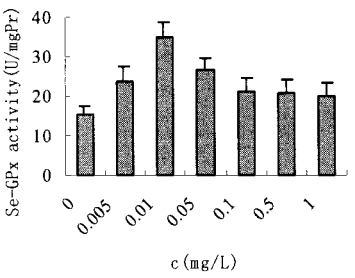

Figure 4. Effects of oil pollution on Se-GPx activity in livers of *Carassius auratus*

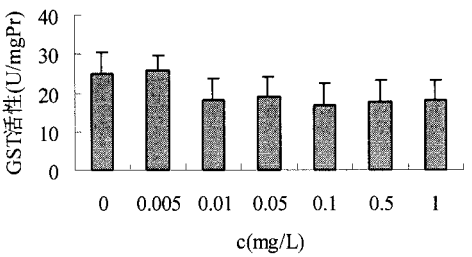

Figure 5. Effects of oil pollution on GST activity in livers of *Carassius auratus*

In short, during 40-d exposure, organisms kept resisting the endogenesis metabolized active oxygen radicals generated by the introduction of extraneous pollutants. This was partially validated in this research by the rapid increase of catalase, SOD, Se-GPx, and GSH synthesis. Catalase, Se-GPx, and GSH were not affected at 0.005 mg/L of oil contamination, while SOD was. Therefore, if the dose-response relationship exists, SOD has potential to be an indicator of oil contamination in an aquatic environment. GST activity was mainly suppressed and its inducibility needs further study.

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