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Immunotoxicity of Tannery Effluent to the Freshwater Fish *Cyprinous carpio*

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Abstract This study was undertaken to determine the effect of chronic exposure to sub-lethal concentrations of tannery effluent (TE) on the humoral antibody response and the cell-mediated immune response of the fish *Cyprinous carpio*. The LC_{50} value of the TE for *C. carpio* was determined by bioassay to be 3.8%. Sub-lethal concentrations of TE (0.6% and 0.3%) significantly suppressed the humoral and cell-mediated immune responses. Exposure of *C. carpio* to the TE had a significant effect on mean acceptance time (MAT) for transplanted scales. MAT was found to be 5–8 days for autografts and 4–7 days for allografts. The somatic indices of the kidney and spleen were reduced compared with controls.

Keywords Humoral and cell-mediated immunity · Tannery effluent · *C. carpio* · Passive haemagglutination · Grafting · LC_{50}

The leather-tanning industry is the major source of chromium deposition in Indian waterways (Khwaja et al. 2001). The uncontrolled release of tannery effluent (TE) to natural water bodies increases environmental pollution and health risks. Semi-aquatic mammals, water birds, and aquatic organisms are exposed directly or indirectly through the food chain to the vast number of chemical compounds contained in this effluent (Mahdavi Talarposhti et al. 2001). Acute toxicity tests have, historically, been important in assessing the effect of human activity on animals, and such tests have wide applicability to evaluating the toxicity of different types and mixtures of pollutants to fish and other aquatic species (Craddock 1977). Immunotoxicity data for chromium are sparse and inconsistent, but indicate that exposure may cause immunosuppression (Arfstein et al. 1998). Exposure of the fish *Saccobuanchus fossilis* to a sub-toxic level of Cr reduced antibody production (Khangarot et al. 1999). The importance of potential damage to aquatic ecology by this effluent has been demonstrated by a variety of tests used in the management of water pollution, for example to estimate environmental effects of waste, to compare the toxicity to animals of different toxicants, and to regulate the amounts of pollutants discharged (Pathan et al. 2009). Exposure of the freshwater cichlid *Oreochromis mossambicus* to chrome-tannery effluents (Sudhan and Michael 1995) and injection of chromium compounds into the body cavity were studied by Arunkumar et al. (2000). The effect of chronic exposure to sublethal concentrations of tannery effluent on the specific immune response and on non-specific immunity in tilapia, *O. mossambicus*, was studied by Prabakaran et al. (2007). Preliminary investigation of the effect of TE (1–15%) on the rate of colonization of plankton was conducted by Koteswari and Ramanibai (2003). Because of widespread contamination of the aquatic environment by TE, there is need for immunotoxicological study to assess the health risks to aquatic animals associated with the effluent. Hence this study focussed on the immunotoxicity of TE to the fish *C. carpio*.

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Materials and Methods

For this study, effluent was collected from a tannery in Erode, Tamil Nadu, India. The effluent was collected in sterile polythene containers and stored at room temperature. Physicochemical properties of the effluent were analysed and are listed in Table 1.

The freshwater fish *C. carpio* was procured from a local fish farm, brought to the laboratory in oxygenated bags, then stored in large cement tanks. The fish were fed with formulated fish feed and acclimated to the laboratory conditions; the water was changed every 48 h. Fish weighing of 28 ± 3 g live weight were used for this study. Ten fish were used for each concentration. Control and replicates were maintained. Each replicate was kept separately in a glass aquarium supplied with water and aeration, and covered with a circular wire-mesh frame. The tests were performed at room temperature (30 ± 2°C). Desired concentrations of effluent were prepared by diluting the mixed effluent with distilled water (Sekar et al. 2009). Raw effluent was regarded as stock solution and standards were prepared by use of distilled water. The acute toxicity test was conducted by exposing the fish in duplicate. Water was renewed every 24 h to avoid depletion of dissolved oxygen, and fresh test media were supplied to maintain a constant concentration of the toxic components. Mortality was recorded for up to 96 h of exposure. The percentage corrected mortality was calculated by use of Abbott's formula (Abbott 1925). The corrected mortality data were analysed to determine LC_{50} values (the concentrations causing 50% mortality). LC_{50} values were obtained by use of a probit regression line, taking test concentrations and the corresponding percentage mortality as log values and probit scales, respectively. By graphical interpolation LC_{50} values were fixed and their fiducial limits (95% upper and lower confidence limits) were also calculated.

C. carpio was exposed to two sub-lethal and safe concentrations, with 1/6th and 1/10th of the 96-h LC_{50} values as high and low sub-lethal concentrations, respectively. Humoral and cell-mediated immunity was analysed for both the high and low sublethal concentrations. At the onset of the experiment (day 0), all groups of fish were immunized with 5 mg S-BSA; on the 30th day the fish were re-immunized, again with 5 mg S-BSA. Each fish was inoculated intraperitoneally by use of a tuberculin syringe (Mafzoub et al. 2009).

Blood samples were collected by caudal vein puncture, by use of a tuberculin syringe. Serum was separated by centrifugation at 3,000 rpm for 10 min, dispensed into clean glass vials, and stored at −20°C. For determination of humoral immune responses, plasma samples were collected after 5, 10, 15, 20, and 25 days for the primary immunization period of the experiment and after 35, 40, 45, and 50 days for the secondary immunization period. Anti-S-BSA antibody titres were determined by use of a microtitre haemagglutination technique (Witlin 1967).

Cell-mediated immunity was determined by the tissue transplantation technique. Experimental fish were placed in anaesthetic (MS 222) at a concentration of 60 mg L^{-1} in water. The fish became quiescent in 20 min. A shallow watch glass or embryo cup with saline was used to retain one scale briefly during grafting. The technique of scale grafting described for goldfish by Hildemann (1957) was followed. In autograft (transplanted within fish) experiments, 5 or 6 scales were taken from the ventral unpigmented regions and transplanted to the dorsal pigmented regions of the same fish. In allograft (transplants between fish from the same population) experiments, fish were randomly paired so they were both recipients and donors. Six scales were plucked from the dorsal pigmented region of the donor fish and kept in saline. From the recipient fish, 5 or 6 scales were removed along a straight line at regular intervals from the ventral unpigmented region. Scales from the saline were inserted singly into the empty dermal scale pockets in the ventral region of the recipient fish. Fish were observed daily for 10 days. Complete acceptance was recorded when there was no sign of rejection or of delayed acceptance, which showed the rejection process was initiated, no sign of melanophore death, and no sign of scale discolouration. Rejection was recorded when the melanophores in the transplanted scales began to disappear, the scale became opaque, and it was eventually sloughed off.

At the end of the experiment the test animals were sacrificed and the spleen and kidney were removed without damaging the organs. Somatic indices were analysed.

Table 1 Physicochemical properties of the effluent

Sample no.	Property	Value[a]
1	COD (mg L^{-1})	4,310 ± 4.51
2	BOD (mg L^{-1})	1,785 ± 4.51
4	TDS (kg m^{-3})	90 ± 2
5	TS (kg m^{-3})	113 ± 2.52
6	Chromium (mg L^{-1})	3,590 ± 2
7	pH	3.36 ± 0.02
9	Turbidity (NTU)	155 ± 0.58
10	Tannin and Lignin compounds (as tannic acid) mg L^{-1}	50 ± 5

[a] ± standard deviation from three replicates

Results and Discussion

The 96-h LC_{50} of the tannery effluent was 3.8% (Fig. 1). In other work, the LC_{50} of the TE to *O. mossambicus* was

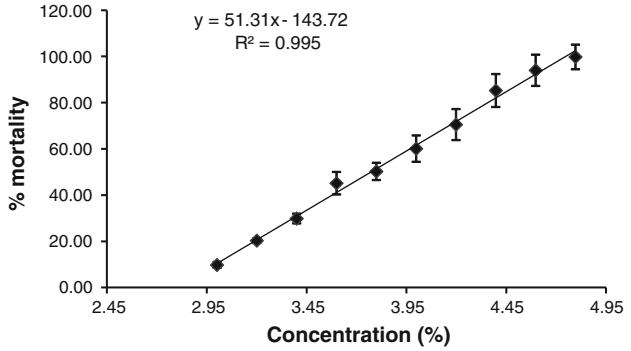

Fig. 1 Mortality to the carp, *Cyprinous carpio*, of paper mill effluent at different concentrations

5.3% (Prabakaran et al. 2007). On the basis of this result, 1/6th and 1/10th of the 96-h LC_{50} were selected as the high (0.6%) and low (0.3%) concentrations, respectively, for sublethal studies.

Figure 2 shows mean antibody titres for all groups of fish after immunization against soluble antigen (S-BSA). Antibody production after primary immunization reached peak values by 15 days for both treated and control fish for both high and low concentrations. The titre then started to decline up to 30 days. After secondary immunization, the antibody titre was gradually increased. At the end of the 50th day antibody production had decreased. These results indicated that the tannery effluent suppressed the primary humoral immune response to BSA administered to fish, and the effect depended on the concentration of the effluent. Chronic exposure to sub-lethal concentrations of tannery effluent had an immunosuppressive effect on *C. carpio*. Similar findings have been reported by Sudhan and Michael (1995). Reduction of haemagglutination titres against SRBC for the freshwater catfish *S. fossilis* exposed to 0.1–3.2 mg L^{-1} of Cr(VI) for 28 days was studied by Khangarot et al. (1999). Chronic exposure of fish to 0.53% TE significantly suppressed the antibody response against

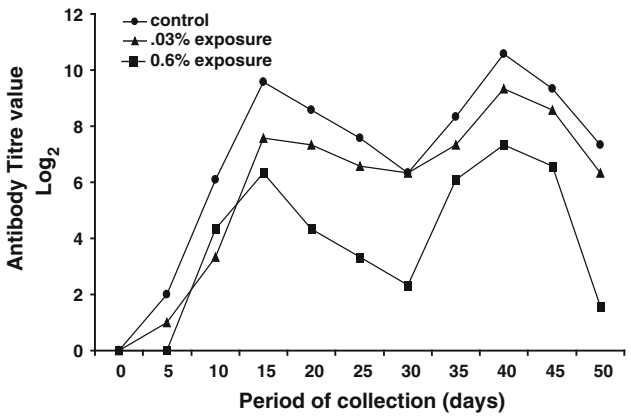

Fig. 2 Effect of exposure of *Cyprinus carpio* to tannery effluent on the antibody response to S-BSA

Aeromonas hydrophilla (Prabakaran et al. 2007). In other work they reported that 0.5 or 5 mg L^{-1} Cr(VI) significantly suppressed antibody titres against heat-killed *A. hydrophilla*. The observed suppression of the secondary humoral immune response may be because of interference of chromium with the structure and function of the memory cells (Snyder and Valle 1991). The suppression of the primary humoral response observed in this study might be mainly because of the toxic effect of the chromium present in the effluent. Chromium has been shown to react with cell-surface receptors for mitogen, block lymphocyte proliferation, and inhibit immunoglobulin production (Borella et al. 1990).

The effect of different concentrations of tannery effluent on the mean acceptance time (MAT) for autografts and allografts were studied. The results are given in the Table 2. MAT was found to be 5–8 days for autografts and 4–7 days for allografts.

The kinetics of cellular immune reactions in cyprinid fish have been extensively studied by transplantation of scales and skin in goldfish and carp, respectively (Lamers Billard and Marcel 1986). In this study, scale grafting was selected because it is a quick, simple, and causes minimum trauma, and because the immune response of fish to scale grafts is relatively well understood (Dawley et al. 2000). According to Hildemann (1957), scale homografts elicit an immune response which can be measured by determination of median survival time and inflammatory reaction under different conditions. In this study, the control fish recognized the grafted scale (autograft) as self and accepted it within 5 days, owing to the sound immune status of the fish. Effluent exposed fish took a long time to recognize and accept. The results showed that even 0.3% tannery effluent was sufficient to impair the immune status of *C. carpio*. In the allografts, the grafted tissue was rejected in a short time because of the antigenic difference. Control fish rejected the grafts much sooner than the exposed fish. This is attributed to the presence of the major histocompatible complex (MHC). Cossarini (2006) reported that no differences were observed for the first and second set of graft rejection times in control and treated (lindane and atrazine) fish. The immunological reaction leading to homograft destruction has been divided into three phases (Billingham

Table 2 Mean acceptance time (days) for control and TE-treated animals

Sample no.	Test animal	Autograft (days)	Allograft (days)
1	Control	5.33 ± 1.37	3.67 ± 1.21
2	0.3%	6.16 ± 1.17	5.5 ± 1.52
3	0.6%	7.67 ± 1.03	7.17 ± 1.48

Values are averages ± standard deviation for ten fish

et al. 1956) involving release of graft antigens (afferent phase), production of antibodies (central phase), and reaction of the antibodies with the graft (efferent phase). In this study, autografts and allografts were more readily rejected by the control fish than the effluent-exposed fish. It was shown that exposure to the tannery effluent impaired the immune system of the fish. Exposure of the freshwater cichlid *O. mossambicus* to chrome tannery effluents (Sudhan and Michael 1995) and injection of chromium compounds into the body cavity (Arunkumar et al. 2000) resulted in spleen atrophy, and reduced leukocyte counts and antibody response on injection of bovine serum albumin. Although lymphocytes and mononuclear phagocytic cells are involved in rejection, the exact mechanisms are still not well understood (Lamers Billard and Marcel 1986). There was a significant difference between control and effluent-treated fish. The high concentration of the effluent was found to result in shorter MAT compared with the control and the low concentrations. WBC count was reduced (data not shown).

The somatic indices of the spleen and kidney were analyzed. The results are given in the Table 3. Spleen and kidney somatic indices were calculated by use of the equations

$$\text{SSI} = \text{Spleen weight (gram wet weight)} \times 100/\text{Total wt of fish (g)}$$

$$\text{KSI} = \text{Kidney weight (gram wet weight)} \times 100/\text{Total wt of fish (g)}$$

Spleen and kidney somatic indices of treated fish were reduced compared with controls. In fish, chromium is accumulated in the kidney, liver, and spleen, in a dose-dependent manner, and those organs are important for antigen-trapping (Khangarot et al. 1999). The SSI% and KSI% indices are indicative of marked concentration-dependent decreases in weight for effluent-treated fish compared with controls. Exposure of the freshwater catfish *S. fossilis* to a subtoxic level of Cr resulted in reduced antibody production, reduced proliferation of splenic lymphocytes, and greater susceptibility to infection by the microorganism *A. hydrophila* (Khangarot et al. 1999).In this study, the fish showed peculiar symptoms at the end of 50th day. The fish developed oedema in the anterior region of peritoneal cavity, convulsions, imbalance in swimming, body twisting, and protrusion of the eye-balls. With increased concentration of the effluent, the symptoms were much pronounced.

Table 3 Somatic indices of kidney and spleen on exposing fish to tannery effluent

Sample no.	Concentration (%)	KSI (%)	SSI (%)
1	Control	0.043 ± 0.005	0.109 ± 0.005
2	0.3	0.032 ± 0.002	0.076 ± 0.006
3	0.6	0.012 ± 0.002	0.045 ± 0.005

Values are averages ± standard deviation for ten fish

From the results of this study it is concluded that any external stressor, even those regarded as non-lethal, can have a detrimental effect on aquatic organisms. This type of study can be useful for comparing the sensitivity of different species of aquatic animals, and the potency of effluent stress can be used as a biological indicator of pollution and as a biological early alarm system for tannery mill effluent.

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