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Effects of effluent from electoplating industry on the immune response in the freshwater fish, *Cyprinus carpio*

V.J. Florence Borgia[a], A.J. Thatheyus[b,*], A.G. Murugesan[c], S. Catherine P. Alexander[a], I. Geetha[a]

[a] PG and Research Centre of Zoology, J. A. College for Women, Periyakulam, Tamil Nadu, India
[b] PG and Research Department of Zoology, The American College, Madurai, Tamil Nadu, India
[c] S.P.K.Centre of Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tamil Nadu, India

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ABSTRACT

The present study was designed to assess the effect of sublethal concentrations of electoplating industry effluent (EIE) on the non-specific and specific immune responses in the freshwater fish, *Cyprinus carpio*. Sublethal concentrations of electroplating industry effluent such as 0.004, 0.007, 0.010 and 0.013% were chosen based on the LC_{50} values. Experimental fish were exposed to these sublethal concentrations of EIE for 28 days. After 7, 14, 21 and 28 days of treatment, non-specific immune response by serum lysozyme activity, myeloperoxidase activity and antiprotease activity and specific immune response by antibody response to *Aeromonas hydrophila* using bacterial agglutination assay and ELISA were assessed. The results showed that chronic exposure of fish to 0.004, 0.007, 0.010 and 0.013% EIE, dose-dependently decreased the non-specific and specific immune responses on all the days tested compared to control fish whereas statistically significant suppressive effects were observed in fish exposed to 0.013% of EIE on all activities tested.

1. Introduction

Aquatic systems are exposed to a large number of pollutants which are mostly released in effluents from industries. Globally, industrial waste water represents the major source of water pollution [1]. Especially waste water from electroplating unit is toxic and hazardous and high in the heavy metals, chromium, nickel, lead, copper and zinc. Tamil Nadu has 400 registered electroplating units operating as small scale sector industries. These units are very small and space is inadequate to have effluent treatment plants. The wastewater may enter public sewer without treatment or aquatic systems or fish farms through agricultural runoff or urban runoff. In certain cases untreated or partially treated or diluted effluents may reach aquatic systems. These pollutants result in severe damage to aquatic life [2]. Toxic substances hinder immune, nervous, endocrine and reproductive systems in animals and their effects may be at organ, tissue and cellular level of organisms [3]. Even very low doses of contaminants can have profound changes on the immune system [4]. Heavy metals are of importance as they alter the immune response by immunostimulatory or immunosuppressive mechanisms [5]. Suppression of immune response may be due to the action of several heavy metals which provide chances for the entry of several pathogens [6]. Immune mechanisms are of significance to fish as they face several environmental challenges. Adverse ecological conditions may have acute or chronic influence on the health status of fish, by changing certain biochemical parameters and by modulating innate and adaptive immune responses [7].

Earlier reports revealed immunosuppression by mercuric chloride on the nonspecific and specific immune system of *Salvelinus namaycush* [8]. Arunkumar et al. [9] observed the effects of chromium on the immune response of *Oreochromis mossambicus*. Witeska and Kosciuk [10] reported that short term zinc exposure induced disturbances in both specific and nonspecific immune mechanisms in common carp. Suppression by paper and pulp mill effluent on the immune functions in *Channa punctatus* (Bloch) was observed by Fatima et al. [11]. Hoeger et al. [12] noticed sewage effluent influencing immune function in *Oncorhynchus mykiss*. Prabakaran et al. [13] reported chromium (VI) exerting stimulatory and suppressive changes on lymphocytes, lysozyme, phagocytic mechanisms and disease resistance in *Oreochromis mossambicus*. Prabakaran et al. [14] also noted decrease in serum lysozyme activity in *Oreochromis mossambicus* due to tannery effluent. Murugesan et al. [15] determined the effect of tannery effluent on the immune response of *Cyprinus carpio*. Rajalakshmi et al. [16] exhibited immunological alterations induced by cadmium and nickel in *Lates calcarifer*. Badr et al. [17] reported that innate immune responses of tilapia could be sensitive to environmental contamination. The effects of industrial effluents were reported by Alexander et al. [4] on the

* Corresponding author.
E-mail address: jthatheyus@yahoo.co.in (A.J. Thatheyus).

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immune responses in fin fish. Changes in immune functions can lead to increased occurrence or severity of infectious diseases. As much work is not done with reference to electroplating industrial effluent, the present study has been designed to perform a battery of assays for both non-specific immune mechanism and specific immunity to ascertain the status of the immune system in *C. carpio* after exposing to sublethel concentrations of electroplating industrial effluent.

2. Materials and methods

2.1. Experimental design

Healthy *C. carpio*, weighing 25 ± 5 g, were purchased and acclimated to laboratory conditions for fifteen days in tanks at a density of 4 g/l. Electroplating industrial effluent was collected one week before starting the experiment from the direct outlets of an electroplating unit near Choolaimedu at Chennai, Tamil Nadu, India, during normal operation period. The effluent was transported to the laboratory in polyethylene containers and filtered to remove the debris and then stored at 4 °C to avoid further activities of microorganisms. The concentrations of chromium (36756 mg/l), nickel (27562 mg/l), lead (11.64 mg/l), copper (335 mg/l), iron (16.48 mg/l) and zinc (206.7 mg/l) were determined by atomic absorption spectrophotometer (Perkin Elmer 2380). The 96 h LC_{50} was determined by a static bioassay [18] following probit analysis [19] as 0.128% of EIE for *C. carpio*. Based on the 96 h LC_{50} value, four sublethal concentrations of EIE such as 0.004, 0.007, 0.010 and 0.013% were chosen and six fish per group (n = 6/group) were exposed for 28 days for immunotoxicity study. In the fish tanks temperature was maintained at 28 ± 2 °C and the sublethal concentrations of effluent were renewed once in every two days. Single tank per treatment was used and all the six fish per group were repetitively bled at regular intervals of 7 days. For each treatment three replicate tanks were used. After 7, 14, 21 and 28 days of exposure, nonspecific immune mechanism in terms of serum lysozyme activity, myeloperoxidase activity and antiprotease activity and specific immune response in terms of antibody response to *Aeromonas hydrophila* using bacterial agglutination assay and ELISA were assessed.

2.2. Serum collection

Six fish per each experimental group as well as control were subjected to bleeding two days before the exposure to EIE and after 7, 14, 21, and 28 days of treatment. The fish were subjected to bleeding serially with 1 ml tuberculin syringe having 24 gauge needle through the common cardinal vein found just below the gills [20]. The total time for bleeding a fish is around 30 s and the fish blood was collected in serological tubes and left for clotting overnight at 4 °C. Then it was centrifuged at 400 g for 10 min to separate the serum. Later the serum was collected in sterile eppendorf tubes and kept at −20 °C until further use.

2.3. Serum lysozyme assay

Serum lysozyme activity was estimated applying turbidimetric assay as narrated by Parry et al. [21] with the microplate adaptation of Hutchinson and Manning [22]. A suspension of 0.3 mg/ml from *Micrococcus lysodeikticus* culture in 0.05M sodium phosphate buffer (pH 6.2) was treated as substrate. Ten microlitres of fish serum (in duplicate wells) was mixed with 250 µl of bacterial suspension in a 96 - well microtitre plate and the decline in absorbance at 450 nm was read after 0.5 and 4.5 min after incubation at 28 °C in a microplate reader (Cyber Elisa-R01, USA). One unit of lysozyme activity was the reduction in absorbance of 0.001 min^{-1} [23].

2.4. Serum myeloperoxidase activity

Serum myeloperoxidase activity was determined following Quade and Roth [24] with slight modification of Sahoo et al. [25]. To 10 µl fish serum, 90 µl phenol red-free Hank's balanced salt solution (HBSS) was added in a 96-well microtitre plate. To this mixture, 50 µl of TMB (3, 3′, 5, 5′, -tetramethyl benzidine hydrochloride)-H_2O_2 (Genei, India) was mixed and incubated for 2 min at room temperature. To arrest the reaction, 50 µl of 2M H_2SO_4 was added and OD was read at a microplate reader at 450 nm against 100 µl of HBSS as blank.

2.5. Serum antiprotease activity

This assay is a slight modification of the method reported by Bowden et al. [26]. It uses aniline-arginine dye ester as a substrate for trypsin, which hydrolyses the aniline dye resulting in a change in colour and it can be determined in a spectrophotometer. Two millimolar BAPNA (sodium-benzoyl-DL-arginine-*p*-nitroanilide HCl, Himedia, India) was the substrate. Ten microlitres of fish serum were incubated with 20 µl of trypsin solution (Trypsin bovine pancreas, 1 mg/ml in 0.01 M Tris HCl, pH 8.2, Himedia, India), in duplicate. Then 500 µl substrate was mixed and the volume was made upto 1 ml with 0.1 M Tris HCl (pH 8.2). It was maintained at 22 °C for 25 min. The reaction was arrested with 30% acetic acid and OD was observed at 415 nm in a plate reader against blank. The inhibitory activity of antiprotease wa recorded as percentage trypsin inhibition as reported by Zuo and Woo [27].

$$\%\text{Trypsin Inhibition} = \frac{\text{Trypsin blank OD} - \text{Sample OD}}{\text{Trypsin blank OD}} \times 100$$

2.6. Antibody titration by bacterial agglutination assay

To detect the effect of electroplating industrial effluent on the specific antibody responses, experimental fish (n = 6/group) were exposed to sublethal concentrations (0.004, 0.007, 0.010 and 0.013%) of electroplating industrial effluent for twenty eight days. After the treatment, fish were administered with heat-killed *A. hydrophila* (10^9 cells/fish) through intraperitoneal injection. An unimmunized control set was maintained under similar conditions. All the exposed fish were repetitively bled regularly at an interval of seven days post immunization till day twenty eight.

Antibacterial antisera were subjected to titration following bacterial agglutination assay adopting the method proposed by Karunasagar et al. [28]. It was carried out in a 96 well "V" bottom microtitre plate. The wells in the rows received 25 µl of phosphate buffered saline. In the first well of a row, 25 µl of sample antiserum was added and two fold serial dilutions were made in that row and the same was repeated in other rows with samples of other antisera. Heat killed *A.hydrophila* suspension of 25 µl (prestained with 0.02% crystal violet) having 10^9 cells/ml was distributed to each well. The microtitre plate was shaken well for proper mixing and incubated overnight at 37 °C. The highest dilution of serum exhibiting detectable macroscopic agglutination was recorded as $\log_2$ antibody titre of the antiserum.

2.7. Enzyme linked immunosorbent assay

Serum antibody levels against *A. hydrophila* were determined by indirect ELISA method narrated by Delamare et al. [29] with slight modifications of Binuramesh et al. [30]. The bacteria were taken from overnight liquid culture and washed thrice with 0.15 mol l^{-1} NaCl solutions and adjusted to 5×10^7 cells ml^{-1} with carbonate bicarbonate buffer pH 9.6. Whole bacterial cells at a concentration of 5×10^7 ml^{-1} were coated with 100µl/well on microtitre plates. The plates were kept at 4 °C overnight and then washed three times with PBS having 0.05% Tween 20 (PBS-T). Nonspecific binding sites were blocked with 100µl/

well of 1% [w/v] BSA in PBS. The plates were incubated at 28 °C for an hour and washed three times with PBS-T. One hundred microlitres of fish serum (test samples) (diluted to 1:30 in PBS-T) in triplicate sets were transferred to wells and kept for 1 h at 28 °C. After incubation, the plates were washed three times with PBS-T and 100 µl of Rabbit anti-carp Ig polyclonal antibody diluted to 1: 10 in PBS was added to each well. The plates were kept for 1 h at 37 °C followed by washing thrice with PBS-T. Then 100 µl of Goat anti-rabbit IgG antibody conjugated with horse radish peroxidase (Sigma, USA) diluted to 1: 2000 in PBS was added to each well and kept for 1 h at 37 °C. The plates were again washed three times with PBS-T. The coloring reaction was developed by mixing 100 µl of 3, 3′, 5, 5′-tetramethyl benzidine - H_2O_2 (Genei India). The reaction was arrested with 25 µl of 1M sulphuric acid and determined at 450 nm using ELISA plate reader. The mean absorbance value for triplicate wells was taken to express serum antibody level.

2.8. Statistical analysis

The data were represented as arithmetic mean ± standard error (SE). Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's posthoc multiple comparison tests. The levels of significance were expressed as *P*-value less or greater than 0.05.

3. Results

3.1. Effect on nonspecific immune response

3.1.1. Serum lysozyme activity

As illustrated in Fig. 1, fish exposed to 0.010 and 0.013% of EIE exhibited statistically significant decrease in serum lysozyme activity on fourteenth, twenty first and twenty eighth days. Statistically significant decline in activity was also observed on twenty eighth day in fish exposed to 0.007% of EIE. Exposure to 0.004% of EIE did not induce any statistically significant ($P > 0.05$) effect compared to control fish on all the days tested and 0.007% of EIE also had insignificant ($P > 0.05$) reduction on seventh, fourteenth and twenty first days.

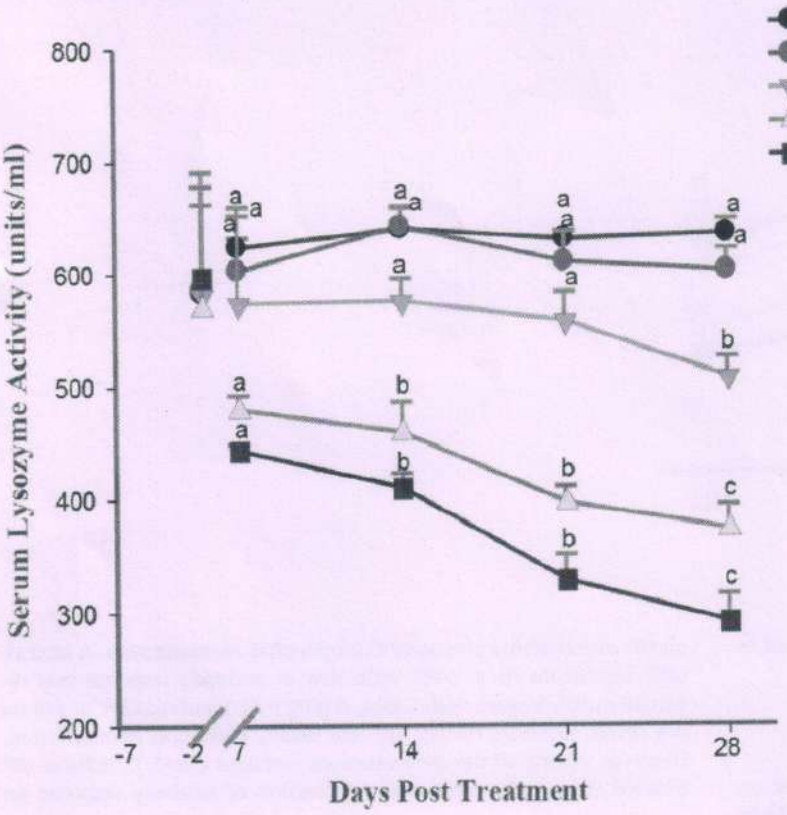

Fig. 1. Effect of chronic exposure to electroplating industrial effluent on the serum lysozyme activity in *Cyprinus carpio*; each point represents mean ± SE of 6 fish; a posteriori Tukey comparison of control and treated groups on individual post treatment days shown with different alphabets representing significant difference ($p < 0.05$).

3.1.2. Serum myeloperoxidase activity

The myeloperoxidase activity showed decline which was statistically significant in all the four concentrations of electroplating industrial effluent than that of control group on day fourteen and twenty eight (Fig. 2). The tested fish exposed to 0.010 and 0.013% of EIE exhibited a statistically significant decrease ($p < 0.05$) in myeloperoxidase activity on day seven, and 0.013% of EIE ($p < 0.05$) exposed fish on day twenty one also. The fish treated with 0.004 and 0.007% of EIE on day seven and twenty one and also the fish subjected to 0.010% of EIE on day twenty one showed decrease in myeloperoxidase activity but the decline was statistically insignificant ($p > 0.05$).

3.1.3. Serum antiprotease activity

Fig. 3 depicts the antiprotease activity in all the fish exposed to 0.004, 0.007, 0.010 and 0.013% EIE being significantly lower than that of the control fish on seventh, fourteenth, twenty first and twenty eighth post treatment days tested. All the concentrations exhibited minimum antiprotease activity on day fourteen than that of the other days (7, 21 and 28) tested. Among the concentrations, the highest concentration (0.013%) of EIE treated group showed maximum suppression of antiprotease activity in all the experimental days tested.

3.2. Effect on specific immune response

3.2.1. Bacterial agglutination assay

The results shown in Fig. 4 decisively indicate that 0.004, 0.007, 0.010 and 0.013% of EIE, dose-dependently suppressed antibody titre on all the days tested after immunization. A statistically significant ($p < 0.05$) decline in antibody response to heat-killed *A. hydrophila* expressed as $\log_2$ antibody titre was found in fish exposed to 0.013% of EIE, whereas the antibody titre showed a statistically insignificant ($p > 0.05$) decline in fish exposed to 0.004, 0.007 and 0.010% of EIE on day seven and twenty eight post immunization. On day fourteen, a peak day, fish exposed to 0.007, 0.010 and 0.013% of EIE showed statistically significant reduction in antibody response. A statistically significant reduction in antibody response was also found in all the

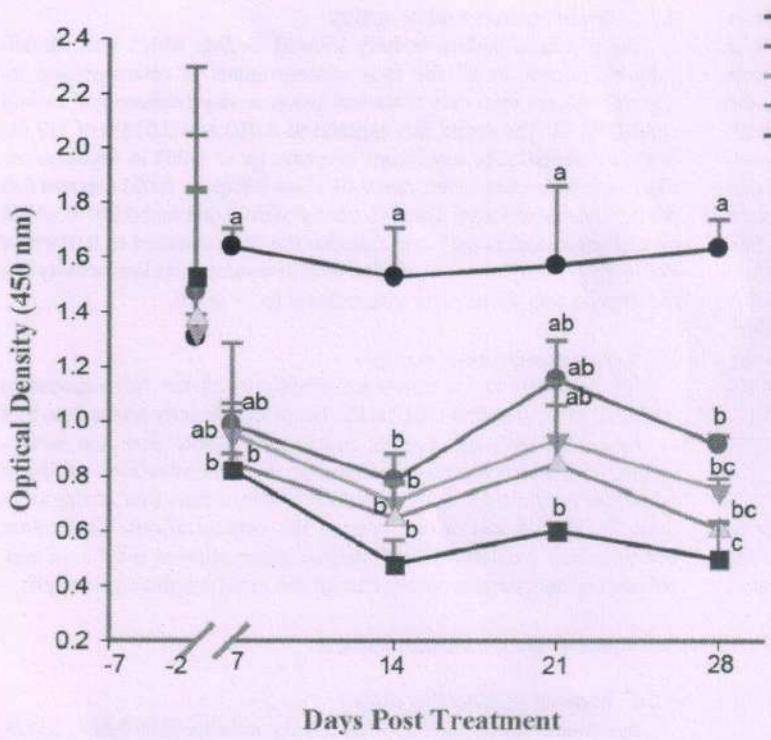

Fig. 2. Effect of chronic exposure to electroplating industrial effluent on the serum meyloperoxidase activity in *Cyprinus carpio*; each point represents mean ± SE of 6 fish; a posteriori Tukey comparison of control and treated groups on individual post treatment days shown with different alphabets representing significant difference ($p < 0.05$).

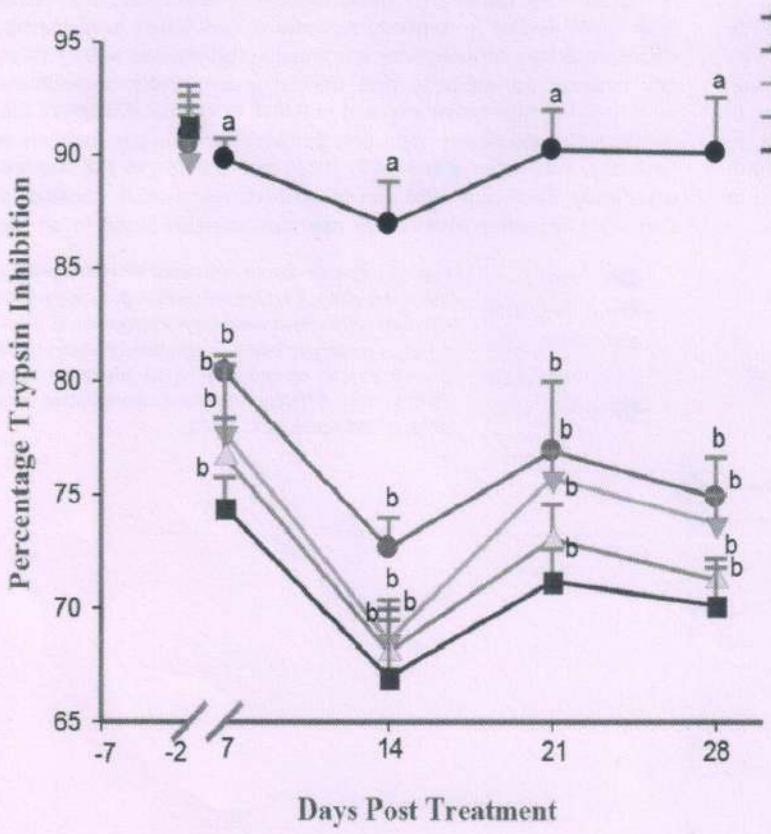

Fig. 3. Effect of chronic exposure to electroplating industrial effluent on the serum antiprotease activity in *Cyprinus carpio*; each point represents mean ± SE of 6 fish; a posteriori Tukey comparison of control and treated groups on individual post treatment days shown with different alphabets representing significant difference ($p < 0.05$).

concentrations (0.013, 0.010, 0.007 and 0.004%) of EIE compared to the control on day twenty one post immunization.

3.2.2. Enzyme linked immunosorbent assay

Fig. 5 illustrates the effect of electroplating industrial effluent on antibody response against heat-killed *A. hydrophila* assayed by ELISA in all the experimental groups of *C. carpio* after immunization. A statistically significant ($p < 0.05$) reduction in antibody response was recorded in fish treated with 0.004, 0.007, 0.010 and 0.013% of EIE on day seven, fourteen, twenty one and twenty eight post immunization. However, among all the concentrations and days tested, 0.013% of EIE induced statistically significant suppression of antibody response on

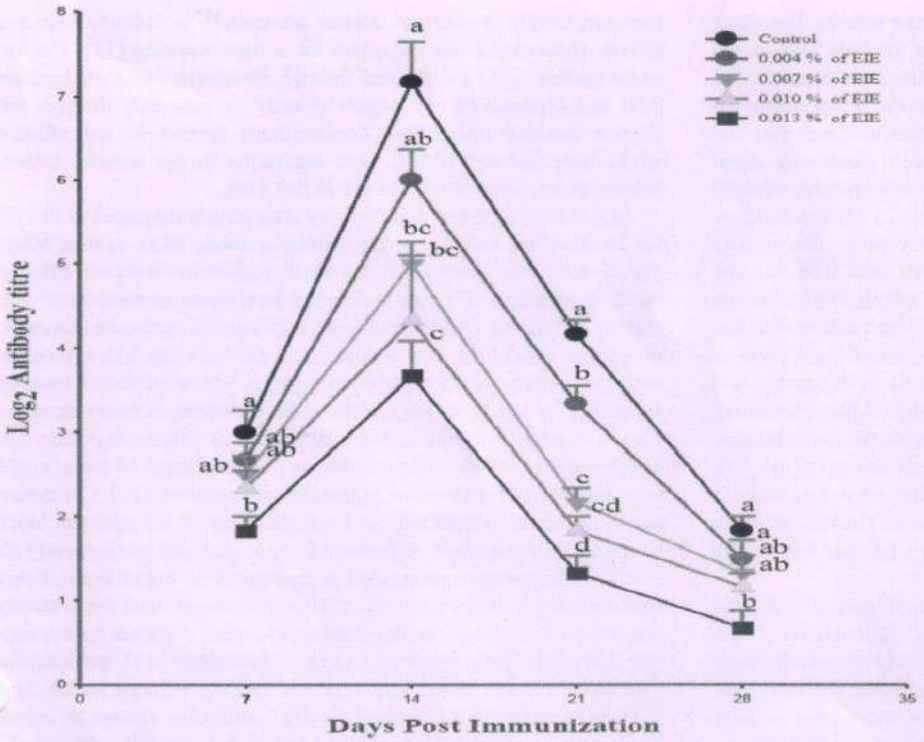

Fig. 4. Effect of chronic exposure to electroplating industrial effluent on antibody response to heat killed *Aeromonas hydrophila* tested by bacterial agglutination assay in *Cyprinus carpio*; each point represents mean ± SE of 6 fish; a posteriori Tukey comparison of control and treated groups on individual post treatment days shown with different alphabets representing significant difference ($p < 0.05$).

day twenty eight after immunization.

4. Discussion

The present investigation indicates that exposure of fish to sublethal concentrations of electroplating industrial effluent suppressed both nonspecific and specific immunity in *C.carpio*. Nonspecific immune mediator lysozyme functioning in blood may be under the influence of stress [31]. Lysozyme activity in fish blood is sensitive to environmental contaminants. Earlier reports explained that decreased level of lysozyme being common in fish exposed to metals. Sanchez-Dardon et al. [32] reported that metal pollution inhibited lysozyme levels and affected regulatory functions in fish. The current work revealed that electroplating industrial effluent reduced lysozyme activity in serum. Chronic exposure of 0.053 and 0.53% of tannery effluent had significant reduction in activity of serum lysozyme in *Oreochromis mossambicus* [14]. Celik et al. [33] noted decrease in the level of lysozyme in zinc exposed *O. mossambicus*. Ghiasi et al. [34] reported decreased level of lysozyme in the common carp, *C. carpio* under cadmium exposure. Rajalakshmi et al. [16] reported reduced lysozyme activity in metal exposed *Lates calcarifer*. Copper toxicity in Javanese carp (*Puntius gonionotus*) [35] and cadmium toxicity in cat fish (*Clarias gariepinus*)

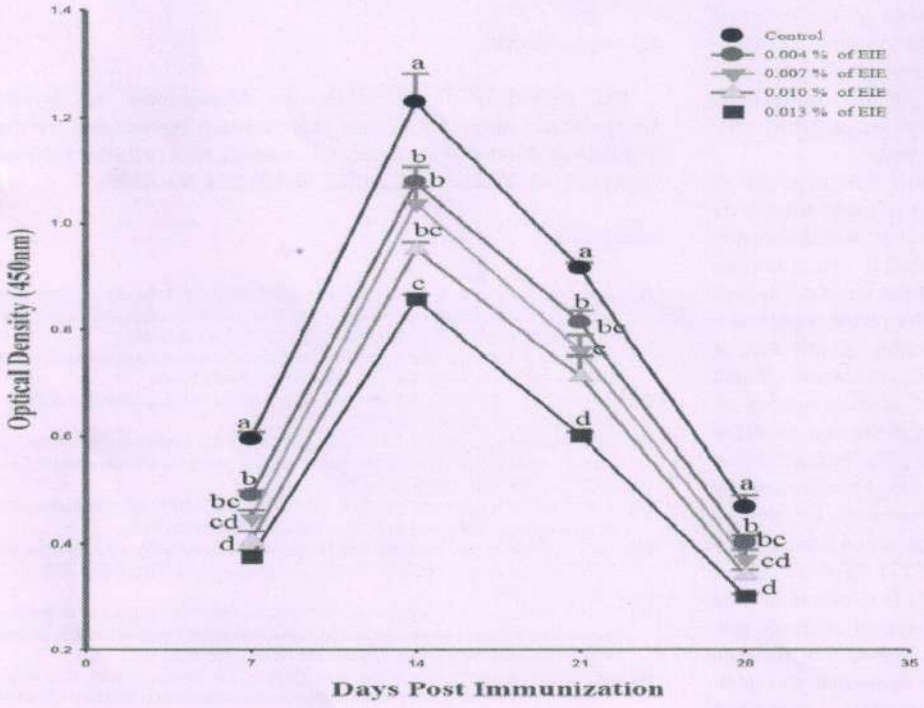

Fig. 5. Effect of chronic exposure to electroplating industrial effluent on antibody response to heat killed *Aeromonas hydrophila* tested by ELISA in *Cyprinus carpio*; each point represents mean ± SE of 6 fish; a posteriori Tukey comparison of control and treated groups on individual post treatment days shown with different alphabets representing significant difference ($p < 0.05$).

[36] were shown to have decreased serum lysozyme activity. Lysozyme levels were insignificantly declined in plasma of the Nile Tilapia, *Oreochromis niloticus* from polluted area in River Basin at Greater Cairo, Egypt [17]. Price et al. [37] exhibited a decline in the activity of serum lysozyme in sewage treated *C. carpio*. Hexavalent chromium significantly decreased serum lysozyme activity with increasing metal concentrations (0.5 and 5 mg/l) in *Oreochromis mossambicus* after 38 days of treatment [13] and (0.1, 1 and 10 µg/l) in *Mytilus galloprovincialis* after 96 h treatment [38]. In the present study also electroplating industrial effluent concentrations (0.010 and 0.013%) significantly reduced the serum lysozyme activity. Metals like chromium are capable of co-ordinate, covalent interaction with macromolecules. Chromium induces oxidative stress through enhanced production of ROS (Reactive Oxygen Species) leading to genomic DNA damage and oxidative deterioration of lipids and proteins. Metals like chromium, copper and molybdenum inhibit lymphocyte proliferation. Metals cause DNA damage in the cells of the immune system and apoptosis [5]. Lysozyme is an important bacterial enzyme of innate immunity and it is involved in the overall alarm response during infection as well as stress. It also acts as an acute phase protein. Mixture of metals was responsible for immunosuppressive effect [16].

Neutrophils are significant in host defense against bacterial, viral and fungal infections [39]. Neutrophils exhibited chemotactic, phagocytic and bactericidal functions, respiratory burst and myeloperoxidase activity in fish [40,41]. Degranulation is necessary for the release of myeloperoxidase, activation of halide production and release of antimicrobial enzymes. Myeloperoxidase uses hydrogen peroxide in respiratory burst to release hypochlorous acid [42]. The activity of myeloperoxidase in granulocytes has to range from 95 to 110 points in normal animals and this value may be from 50 to 70 points in toxicant exposed animals [43]. Reduction in myeloperoxidase activity indicates the exposure to contaminants or stress [44]. In the current study myeloperoxidase activity was decreased in *C. carpio* exposed to electroplating industrial effluent. Zinc had an immunosuppressive effect on fish [45]. Similar result has been reported in *Oreochromis mossambicus* exposed to 5 ppm zinc [33].

Fish plasma has many protease inhibitors, mainly α1-antiprotease, α2-antiplasmin and α2-macroglobulin which play a key role in restricting the character of bacteria to invade and multiply *in vivo* [46]. Protease inhibitors can stop replication of pathogens without unexpected toxicity to the host [47]. The study with all the sublethal concentrations of electroplating industrial effluent showed an inhibition of antiprotease activity in *C. carpio*. The observations of the present work display that sublethal doses of electroplating industrial effluent do suppress the activity of natural antiprotease in fish serum, which may not offer defense against pathogens entering the body.

The observations on antibody response show that exposure to electroplating industrial effluent dose-dependently suppressed antibody titre against heat–killed *A. hydrophila* on all the days tested after immunization. Chronic exposure to all the sublethal concentrations showed significant suppression of antibody response on 21st day post immunization. Similar trends were reported earlier on the suppression of both primary and secondary immune responses against BSA in *Oreochromis mossambicus* treated with 2.5–10% LC_{50} of tannery effluent after 21 days [48]. Treatment with 0.53% and 0.053% of tannery effluent on day 14 and 0.53% of tannery effluent on all the days exhibited suppression of antibody titre in *O. mossambicus* [14]. Intraperitoneal injection of chromium (10, 1, 0.1, and 0.01% LD_{50}) suppressed the humoral immune response against BSA in *O. mossambicus* [9]. Similar results were reported on the decline of hemagglutination titers against sheep RBC in *Saccobranchus fossilis* treated with 0.1, 1.0 and 3.2 ppm of chromium after 28 days [49]. Prabakaran et al. [13] examined that exposure of chromium at 0.5 and 5 mgl^{-1} decreased antibody production against heat killed *A. hydrophila*. They opined that the suppression might be because of the interaction of chromium with lymphocyte surface proteins. Hexavalent chromium suppressed immunoglobulin production. Metals accumulate in kidney, liver and spleen. These organs are important for antigen trapping [14]. The immune system could be affected through disruptive effect on immune cells and immune related organs directly or indirectly through endocrine–immune interaction. Contaminants present in the effluent might have induced HSP70 gene expression in the muscle, spleen, kidney, heart, liver, brain and gill of fish [14].

Sugatt [50] reported that *Oncorhynchus kisutch* subjected to Cr (VI) for 14 days had reduced agglutinating antibody titers against *Vibrio anguillarum*. The presence of bacterial agglutinins indicate either a previous exposure to disease or contact with water contaminated with certain pathogens, which cross react with natural antibodies found in the serum of fish [25]. The finding in the present work is in agreement with the reports in *Trichogaster trichopterus* which exhibited lowered production of agglutinating specific antibodies after mercury exposure [51]. A combination of B cell activation due to effluent exposure and antigen and elevated cortisol levels as a consequence of stress could have resulted in the decrease in lymphocyte numbers [12]. Chromium has been shown to react with cell surface receptors for mitogen, block lymphocyte proliferation and inhibit immunoglobulin production [13].

The immune response against *A. hydrophila* by ELISA showed that electroplating industrial effluent significantly suppressed the antibody responses in *C. carpio*. Similar results have been reported on the suppression of antibody response against *A. hydrophila* in *O. mossambicus* exposed to 0.0053, 0.053 and 0.53% of tannery effluent for 28 day [13]. In contrast, ELISA analysis for IgM antibodies against *A. salmonicida* was not affected by sewage treatment in *Oncorhynchus mykiss* [12]. A single mechanism cannot be attributed for the immunotoxic effects of all heavy metals and these metals may affect the cells of the immune system in several ways. Metals when present in a mixture also compete for intracellular binding sites [32].

5. Conclusion

The fish mainly depend on their nonspecific and specific immune responses. In the present work, the suppression in nonspecific immune factors such as lysozyme activity of serum, myeloperoxidase activity and antiprotease activity and specific immune response through antibody level were observed after exposure to electroplating industrial effluent and such fish may be prone to any infection.

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