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MATERIALS AND METHODS

The common carp Cyprinus carpio is a freshwater teleost fish belongs to the family Cyprinidae is chosen for the present study because of the following reasons,

1. Fast growth
2. Highly nutritious
3. Table value
4. Resistance to disease and
5. Easily adaptable to laboratory conditions

Collection and Maintenance
Cyprinus carpio (20 ± 2gm) of both sexes were procured from Tamil Nadu Fisheries Development Corporation Ltd. (Fish farm) Mettur, Tamil Nadu, India and were brought to laboratory without any mechanical injury and washed with 0.1% KMnO₄ solution to remove dermal infection, if any, and were acclimatized in a large cement tank to the laboratory conditions (24.2°C) for one month. Fish fed ad libitum with commercial fish feed (Hallow fish feed) once a day. A major portion of water was changed daily in order to avoid any accumulation of excretory products and unused feed, which may cause further stress to the fish.

Toxicant
The hexavalent chromium (potassium dichromate K₂Cr₂O₇) (MERCK, Mumbai, India, Purity 99 % w / w) was used as the toxicant. The stock solution was prepared by using double distilled water and the required concentrations were prepared from the stock solution.

Physico - Chemical Properties of Hexavalent chromium
a) Chemical Formula K₂Cr₂O₇
b) Molecular weight 294 g / mol
c) Density 2.676 g / cm
d) Colour Red - Orange crystalline solid
e) Melting point 398°C  
f) Boiling point 500°C  
g) Odour Odourless  
h) Specific gravity 2.68

It is soluble in water and slightly soluble or insoluble in ethyl alcohol. It acts as a powerful oxidizing agent. It exists in many states of oxidation, ranging from +2 to +6, forms +3 e +6 are the most stable in the environment.

Chemical structure of Hexavalent chromium

Water Quality and Analytical Techniques

Dechlorinated tap water was used for the experiments. Since, physico-chemical parameters like temperature, pH, salinity, dissolved oxygen, total hardness and total alkalinity of the water have significant influences on the biodegradability and toxicity of pollutants, physical and chemical parameters were analyzed (APHA, 2005) for each set of experiment and the data were tabulated in Table No. 1 and were maintained throughout the study period. There were no significant variations in the values. They were maintained in glass aquaria containing dechlorinated water (pH 7.1 ± 0.1, Dissolved oxygen 7.4 ± 1.0 mg / L, total hardness 18.1 ± 0.4 mg / L as CaCO₃ and alkalinity 21.1 ± 10.1 mg / L as CaCO₃ under natural photoperiod (13 L:11 D). Feeding was discontinued 24 hours before the commencement of the experiments to keep the test animals in more or less same state of metabolic requirements (Reish and Oshida, 1987).
Table No. 1. Physico chemical parameters of the water used for the investigations

<table>
<thead>
<tr>
<th>S. No</th>
<th>Factors</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Temperature</td>
<td>26.0 ± 2°C</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>3.</td>
<td>Salinity</td>
<td>0.6 ± 0.1 ppm</td>
</tr>
<tr>
<td>4.</td>
<td>Dissolved oxygen</td>
<td>7.4 ± 1.0 mg/L</td>
</tr>
<tr>
<td>5.</td>
<td>Total hardness</td>
<td>18.1 ± 0.4 mg/L</td>
</tr>
<tr>
<td>6.</td>
<td>Total alkalinity</td>
<td>21.1 ± 10.1 mg/L</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM of 5 observations.

Determination of Median lethal concentration

One gram of potassium dichromate (Hexavalent chromium) was transferred to a one litre standard flask; double distilled water was added up to the mark and the toxicant were dissolved well. From this stock solution, appropriate quantity was taken and dissolved in tap water to get the desired concentrations of the metal solution.

Preliminary toxicity tests were carried out to find out the median lethal tolerance limit (LC50) of the fish to the toxicant chromium for 96 h. The concentration at which 50 % mortality/survival occurred after 96 h was taken as the median lethal concentration for 96 h.

The LC50 value of chromium was determined by using daily renewal bioassay system. The test concentrations were chosen on the basis of initial experiments to determine the LC50. For the determination of LC50, 10 fish were introduced into each set with gradually increasing concentrations of Cr (VI) (potassium dichromate) with the interval of 10 mg simultaneously. Experimental period for each concentration was 96 hours. The toxicants in the test chamber was renewed completely with fresh solution daily and the fish were fed with commercial fish feed (Hallow fish feed) once a day. In the experiment no distinction was made between the sexes. The control
group without hexavalent chromium was maintained simultaneously. Mortality of fish was recorded in each concentration till 96 h LC$_{50}$ value was calculated by following Finney’s probit analysis (1971). Triplicates were maintained for both control and experimental sets. Aeration was provided with automatic compressor and unconsumed food was removed every day.

Experimental Design

Two experimental series were performed viz. acute and sublethal hexavalent chromium toxicity studies. In each experimental study, control and replicates of treatment groups were maintained.

Acute Toxicity Studies

After determining LC$_{50}$ for 96 h, healthy fish of both sex were stocked in a large cement tank (1200 lit) after it was cleaned and disinfected with potassium permanganate. Fish with an average weight (20 ± 2 gm) were selected for the experiment. The acute experiment was carried out for 96 hours in 5 circular plastic tubs, each filled with 200 L of water. A normal pH (7.1) and hexavalent chromium concentration of 59.26 mg / litres (96 h LC$_{50}$ concentration) were maintained throughout the experiment. 10 fish were introduced into each tub. Control was maintained in circular plastic tubs with 10 fish per tub. The fish were fed ad-libitum with commercial feed once in a day. A facility of oxygen was provided.

Sampling Frequency

At the end of 96 h, fish from the control and treatment tubs were taken for analysis. A minimum of 10 fish per treatment (5 replicates per treatment and 10 fish per replicate) were used for acute toxicity studies. Care was taken to minimize disturbances to the experimental animals.

Sublethal Toxicity Studies

Sublethal effects of (1/10$^{th}$ of concentration of 96 h LC$_{50}$) hexavalent chromium toxicity were studied in 500 L plastic tubs, filled with clean dechlorinated water. The tubs were labeled with ‘C’ and ‘T’ represented control and experiment, respectively. Tub ‘T’ was filled with sublethal concentration of hexavalent chromium

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(5.93 mg / L, ie. 1/10th of 96 hr LC50, 59.26 mg / L) as suggested by Sprague (1971) and tub ‘C’ was filled with 200 l clean dechlorinated water. Then 100 fish of both sexes were randomly selected from the stock and allowed to the plastic tub, 100 in each group, and 100 in each replicate. Care was taken to minimize disturbances to the animals. For each acute and chronic exposure, replicates were maintained.

Exposure and Maintenance

During sublethal studies, fish were fed ad libitum with commercial feed once in a day before water replacement. Since, the tests were carried out under static conditions, it was necessary to transfer fish to fresh solution in order to reduce any accumulation of excretory products and left over feed which may cause further stress to the fish. Water was changed daily and redosed again. An oxygen facility was provided for the test solution.

Sampling Frequency

Sublethal experiments were carried out for 32 days (8, 16, 24 and 32 days). At the end of eight day, 25 fish were randomly selected from control and experimental groups and sacrificed without being anaesthetised for analysis. Blood was taken from caudal vein using plastic disposable syringe fitted with 26 gauge needle which was already moistured with heparin as an anticoagulant. Blood collected from treatment and control was expelled into the separate heparinized plastic vials and placed immediately on ice. Pooled blood sample was used for determination of all the parameters.

Sample Preparation

Plasma

All the analyses were performed on pooled blood samples from fish collected after identical exposure. Blood was centrifuged for 15 minutes at 10,000 rpm and plasma was withdrawn and transferred into clean vials for further analysis viz. glucose, cholesterol, protein, lactic acid, GOT, GPT, LDH and GSH.
Tissue

After drawing blood, fish were washed with double distilled water and blotted dry with absorbent paper. Then the fish were cut open and gill, liver and kidney were removed, stored in respective plastic vials for accumulation and histological studies.

Accumulation Studies

Tissue Preparations for Accumulation Studies

After the exposure of fish to acute and sublethal chromium toxicity studies for different exposure periods (8th, 16th, 24th and 32 days), fish were sacrificed, washed with double distilled water and blotted dry with absorbent paper. Then the fish was cut opened, gill, liver and kidney tissues were removed according to method of Dybem, (1983) and stored in respective plastic vials for further accumulation studies.

All the glasswares used for the experiment were rinsed for 12 hours with 5 N Hydrochloric acids and subsequently rinsed thrice in double distilled water to remove any metal contamination. Plastic implements were used to isolate the organs.

Digestion Procedure

From the pooled samples 0.5 g of gill, liver and kidney were weighed into 100 ml silica flasks respectively and wet digested with analytical grade concentrated nitric acid followed by concentrated perchloric acid and in the ratio of 3:1 (v/v). On completion of digestion, the acid solution was reduced in volume of about 1 to 2 ml, transferred to a 25 ml graduated flask and made upto the mark with deionized water. The solutions were then analysed by assayed using Shimadzu AA 6200 Atomic absorption spectrophotometer.

Haematological Studies

Total red blood corpuscles (RBCs) were counted using an improved Neubaur haemocytometer (Shah and Alttindag, 2004a). Total white blood corpuscles (WBC) were counted using an improved Neubaur haemocytometer (Mgenka et al., 2003). Haemoglobin (Hb) was determined with a haemoglobin test kit (DIAGNOVA,
Ranbaxy, India) using the Cyanometemoglobin method (Blaxhall and Daisley, 1973). Hematocrit level was estimated by using Wintrobes tube method (Ochei and Kolhatkar, 2005).

Biochemical Studies

Plasma glucose was estimated by O-Toluidine method (Cooper and Mc Daniel, 1970) using glucose kit supplied by Span Diagnostic pvt. Ltd. Surat, India. Plasma protein was estimated according to the method of Lowry et al. (1951). Plasma cholesterol was estimated by the method of Wybenga and Pileggi (1970) using Span Diagnostic Pvt, Ltd, Surat, India. Plasma Lactic acid was estimated by the method of Barker and Summersor (1941).

Enzyme Studies

Plasma GOT, GPT, LDH and GSH activities were estimated in the common carp, C.carpio exposed to acute and sublethal hexavalent chromium toxicity at the end of stipulated experimental periods. The activities of blood GPT and GOT were estimated according to the colorimetric method of Reitman and Frankel (1957). The enzyme lactate dehydrogenase activity was estimated by the method of King (1965). Reduced glutathione was estimated by using Ellman’s method (1959).

Histological Studies

At the end of acute and sublethal hexavalent chromium toxicity for various exposure periods (8th, 16th, 24th and 32nd days), fish of all the groups were sacrificed by decapitation. Gill, kidney and liver of control and treated fish were dissected out, and histological studies were carried out by Cullling method (1974), sectioned at 7 μm thickness and stained with Haematoxylin–Eosine (Bancroft et al., 1996). The histological changes in the tissues were examined in the randomly selected sections from each fish. Histological changes induced by treatments in the tissues were photographed using Nikon photomicroscope.
Determination of median lethal concentration

Survival / mortality of fish in the control and experimental tubs after 96 h were recorded. The concentration at which 50 % survival /mortality occurred after 96h was taken as median lethal concentration (LC\textsubscript{50} ) for 96h. The central point (LC\textsubscript{50} narrow range) was repeated four times to arrive at the median lethal concentration. The LC\textsubscript{50} value for 96 h was determined by probit analysis method of Finney (1971). Homogeneity of the population used in the present investigation was tested using chi-square test according to Busvine (1971).

Statistical Analysis

The results were subjected to statistical analysis (SPSS) and data were expressed as mean ± SEM. The differences between mean values were evaluated by one way analysis of variance (ANOVA), statistical significance of the differences was assessed by using Students-t test and significance was established at P<0.05 level.