

Persistent sub-lethal chlorine exposure augments temperature induced immunosuppression in *Cyprinus carpio* advanced fingerlings

A.K. Verma^a, A.K. Pal^{b,*}, S.M. Manush^b, T. Das^b, R.S. Dalvi^b,
P.P. Chandrachoodan^c, P.M. Ravi^d, S.K. Apte^e

^a College of Biotechnology, Allahabad Agricultural Institute (Deemed University), Allahabad 211007, Uttar Pradesh, India

^b Fish Biochemistry Laboratory, Central Institute of Fisheries Education, Fisheries University Road, 7 Bunglows, Versova, Andheri (W), Mumbai 400061, India

^c Board of Research in Nuclear Sciences, Department of Atomic Energy, Government of India, India

^d Environmental Survey Laboratory, Kaiga, Karnataka, India

^e Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai, India

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Abstract

Apart from increased temperature, thermal effluents discharged through cooling systems of nuclear power plants may often contain chlorine (used against bio-fouling), which may affect the immune status of fish. Therefore, a 28-day trial was undertaken to delineate the effect of high temperature and a persistent sub-lethal chlorine exposure on immunomodulation in *Cyprinus carpio* advanced fingerlings. Fish were acclimated to four different temperatures (26, 31, 33 and 36 °C) and maintained for 30 days in two different groups. One group was exposed to persistent chlorine (0.1 mg L⁻¹) and was compared with their respective temperature control groups (without chlorine exposure). Expression of heat shock proteins (hsp 70) was tested in muscle after 28 days using Western blotting. Haematological parameters (erythrocyte count, leucocyte count, haemoglobin), serum parameters (total protein, albumin, globulin, A/G ratio) and respiratory burst activity were tested to assess immuno-competence of *C. carpio* in response to temperature and chlorine exposure. Results indicated that hsp 70 was induced at 36 °C in temperature control groups but not in their respective temperatures in the presence of chlorine. Haematological parameters such as haemoglobin, erythrocyte and leucocyte counts appeared depressed in chlorine treated groups as compared to their respective temperature control groups. Serum protein and globulin were affected due to chlorine exposure at different acclimation temperatures. A decrease in NBT activity was recorded in chlorine treated groups as compared to their respective temperature control groups. Overall results indicate that increasing acclimation temperatures alters the immune status of *C. carpio* advanced fingerlings and persistent sub-lethal exposure to chlorine augments this temperature induced immunosuppression.

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* Corresponding author. Fax: +91 22 26361573.

E-mail address: akpal_53@rediffmail.com (A.K. Pal).

1. Introduction

In a healthy state, fish defend against potential invaders with a complex system of innate and adaptive immune mechanisms. In spite of limited pathogen recognition machinery, the strength of innate defence mechanisms against biotic and abiotic stressors is impressive [1]. Stressors may directly kill the fish or indirectly exacerbate diseased state by lowering the resistance and allowing the invasion of environmental pathogens [2]. Exposure to individual stressors may affect the immune system in a variety of ways; altering macrophage function [3,4] and circulating levels of immune cells. Ambient water temperature is critical in the development of both specific and non-specific immunity in fish [5]. Increasing temperature up to a certain limit favours fish growth by increasing the metabolic activities [6,7]. However, elevated water temperature (within the physiological range of fish) has been shown to alter the immune function [8]. For example, exposure of Catfish (*Heteropneustes fossilis*) to elevated temperature increases mitochondrial superoxide (O_2^-) production in the gills [9] and enhances antibody activity in Atlantic cod, *Gadus morhua* L. [10]. Further, beyond the range of preferred temperature zone, fish become more vulnerable to diseases due to metabolic injury, immunosuppression, carcinogens, etc. In addition to temperature, exposure to xenobiotics may act synergistically in causing immunosuppression in fish. However, the effect of multiple stressors on immunomodulation in fish still remains elusive.

Thermal discharges from nuclear power plants often contain chemical stress factors in the form of different biocides, in addition to high temperature. Amongst these, chlorine is widely used for bio-fouling control [11]. Chlorine is added to cooling effluent waters to neutralize mussel, algae and other marine fouling populations [12] in the immediate vicinity of the power plant, as growth of these aquatic organisms may hamper the flow of cooling waters to the condensers. For efficient operation of nuclear power plants, uninterrupted supply of cooling water to the condensers is a prerequisite [13]. One of our preliminary investigations indicated that the evaporation rate of chlorine increases with increasing temperatures (data unpublished). However, a steady level of chlorine is maintained in cooling condensers of nuclear power plants by continuous supplementation of chlorine at the intake point. Condenser effluents thus may have the potential to impart thermal and chemical stress on living organisms [14]. Continuous use of chlorine may thus affect non-target organisms by diffusing through their cell membrane, and inhibiting various metabolic activities.

There have been no reports available on the combined effect of persistent exposure to high temperature and chlorine on the immune status of fish. Therefore, in our study, *Cyprinus carpio* advanced fingerlings were selected to assess the effect of increasing temperature and a sub-lethal (0.1 mg L^{-1}) level of chlorine. *C. carpio* can tolerate a wide range of temperature ($13\text{--}42^\circ\text{C}$), as per our earlier investigations in early fingerlings [15], which may be the reason for their cosmopolitan distribution. The temperatures chosen ($26, 31, 33$ and 36°C) in the present study were therefore well within the range of the test fish and a variety of carps, including Indian Major Carps [16]. A panel of assays considered pertinent in our study for assessing fish health includes RBC, haemoglobin, WBC and plasma protein values [36,37]. Respiratory burst activity has been considered as a critical effector mechanism in neutralising the biotic stressors [17]. Heat shock proteins (hsps), a class of acute phase proteins secreted in response to a variety of stresses, [18–20] were also tested.

2. Materials and methods

2.1. Experimental fish

C. carpio (mean \pm SE: 11.13 ± 0.55 g) were brought in open aerated containers from Khopoli fish farm, Government of Maharashtra, to the wet laboratory, Central Institute of Fisheries Education, Mumbai, and were acclimatized for 30 days to laboratory conditions. Fish were fed with supplementary diet (25% crude protein) before being subjected to acclimation trials.

2.2. Chlorine dosage and analysis

As per earlier toxicity studies, LC_{50} of chlorine in *C. carpio* (average weight $8\text{--}10$ g) was recorded as $0.4\text{--}0.5 \text{ mg L}^{-1}$. Therefore, a sub-lethal concentration ($1/5$ th of LC_{50}) was selected for our study. A preliminary experiment was carried out to assess the evaporation rate of chlorine with respect to different temperatures and monitored

at 8-h intervals. Based on the evaporation rate, chlorine was supplemented to maintain the level at $(0.1 \pm 0.02 \text{ mg L}^{-1})$ in experimental tanks. HPLC grade hypochloride solution was used as the chlorine source. Chlorine levels were continuously monitored by using Spectroquant Chlorine test kit (1.00 599.0001, E Merck, Germany) with an accuracy of 0.01 mg L^{-1} .

2.3. Acclimation of experimental fish

A total of 72 fish were acclimated to four different temperatures (26, 31, 33 and $36 \text{ }^\circ\text{C}$) and maintained in two different groups (nine fish per tank). One group was exposed to persistent chlorine (0.1 mg L^{-1}) and was compared with their respective temperature control groups (without chlorine exposure). Acclimation was carried out in thermostatic aquaria (175 L water capacity, sensitivity $\pm 0.2 \text{ }^\circ\text{C}$) at the rate of $1 \text{ }^\circ\text{C/day}$ from laboratory temperature ($26 \text{ }^\circ\text{C}$) to reach experimental temperatures (26, 31, 33 and $36 \text{ }^\circ\text{C}$). After successful acclimation, 0.1 mg L^{-1} of chlorine was maintained in one group throughout the experimental phase (28 days). All the fish survived till the end of the exposure period.

2.4. Sampling and analysis of hsp 70 expression

At the end of 28 days of exposure, three fish from each group were anaesthetised using CIFECALM ($50 \mu\text{L L}^{-1}$). CIFECALM is a herbal anaesthetic formulation containing natural alcoholic extracts of *Eugenia caryophyllata*, and *Mentha arvensis* (developed by Central Institute of Fisheries Education, Mumbai) and tissue homogenate (20% w/v) was prepared using freshly dissected muscle of individual fish (0.5 g approx. in triplicates from each group), using Tris-buffered saline (pH 7.5) under chilled conditions with protease inhibitor, 0.1 mM phenyl methane sulfonyl fluoride (PMSF). Homogenate was centrifuged ($3000 g$ at $4 \text{ }^\circ\text{C}$ for 10 min). Supernatant was collected and preserved frozen ($-20 \text{ }^\circ\text{C}$) for further hsp 70 analysis by Western blotting [21]. Thawed sample supernatant was analyzed for total protein content [22]. Sample buffer was immediately added to each sample and heated to $95 \text{ }^\circ\text{C}$ for 2 min. Sub samples of protein ($50 \mu\text{g}$) were separated by SDS-PAGE with 12% separating and 5% stacking polyacrylamide gels [23], using an electrode buffer [24]. Hela cell lysate (heat shocked, Bioreagents-LYC-101 F, Stressgen, Canada) ($20 \mu\text{g}$) was loaded onto a lane to serve as an internal standard for assessing the blotting efficiency. Proteins were separated at 1.5 mA per well for approximately 3 h and then electro-blotted on to a Polyvinylidene fluoride (PVDF) transfer membrane (E578-10 \times 10 cm SQ, USA) at 200 mA for 10 h. After blotting, gels were stained with Coomassie blue to ensure complete transfer. Membranes were blocked with 3% Bovine Serum Albumin and Tris-Buffered Saline (TBS pH 7.4). Tween 20 (0.05%) in TBS was used as a washing solution. Primary monoclonal antibodies against carp hsp 70 (1:2000 dilution, Bioreagents-SPA-810, Stressgen, Canada) were used as probes. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution, Bioreagents-SAB-100, Stressgen, Canada) was used to detect hsp 70 probes. Finally, the membrane was incubated in a colour developing solution containing diaminobenzene and H_2O_2 . Bound antibodies were visualized by Gel Documentation system (Syngene, UK).

2.5. Blood collection

At the end of the trial, fish (in triplicates) from each group were anaesthetised (as described above) and blood was collected from the caudal vein using a syringe, which was previously rinsed with 2.7% ethylenediamine tetraacetic (EDTA) (as anti-coagulant) and transferred immediately into a test tube containing EDTA. Blood collected was used for determination of haemoglobin content, total erythrocyte and leucocyte count and NBT assay. For serum separation, the remaining three fish from each group were anaesthetised and blood was collected without anti-coagulant, and allowed to clot for 2 h, centrifuged ($3000 \times g$ for 5 min) and stored at $-80 \text{ }^\circ\text{C}$ until further analysis.

2.6. Haematological parameters

2.6.1. Haemoglobin content

The haemoglobin level of blood was analyzed following the cyanmethemoglobin method using Drabkin's Fluid (Qualigens Diagnostics). The absorbance was measured using a spectrophotometer at 540 nm and the final

concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Diagnostics). The haemoglobin concentration was then calculated by using the following formula:

$$\text{Haemoglobin (gm/dl)} = [\text{OD(T)}/\text{OD(S)}][251/1000] \times 60$$

where OD (T) = absorbance of test and OD (S) = absorbance of standard.

2.6.2. Total erythrocyte and leucocyte counts

Red blood cells (RBC) and white blood cells (WBC) diluting fluids were used to determine the total erythrocyte and leucocyte counts. It was done by mixing 20 μl of blood with 3980 μl of corresponding diluting fluid in a clean test tube and shaking well. Cell counts were performed using a Neubauer's counting chamber.

2.7. Serum parameters

Serum protein was estimated by Biuret and BCG dye binding method [25] using a kit (total protein and albumin kit, Qualigens Diagnostics, Division of Glaxo Smithkline Pharmaceutical Limited). Albumin was estimated by the bromocresol green binding method [26]. The absorbance of standard and test was measured against a blank in a spectrophotometer at 630 nm. Globulin was calculated by subtracting albumin values from total serum protein. A/G ratio was calculated by dividing albumin values by globulin values.

2.8. Respiratory burst

The respiratory burst activity of the phagocytes was done by nitroblue tetrazoleum (NBT) assay following the method of Secombes [27] subsequently modified by Stasiack and Bauman [28] similar to our earlier investigations [29]. Blood (50 μl) was placed into the wells of 'U' bottom microtitre plates and incubated at 37 °C for 1 h to facilitate the adhesion of cells. Then the supernatant was removed and the wells were washed three times in PBS. After washing, 50 μl of 0.2% NBT was added and incubated for a further 1 h. The cells were then fixed using 100% methanol for 2–3 min and washed three times with 30% methanol. The plates were then air-dried. Sixty microlitres of 2 N potassium hydroxide and 70 μl dimethyl sulphoxide were added. The optical density was recorded in an ELISA reader at 540 nm.

2.9. Statistical analysis

Data obtained from the experiments were tested for significance using one-way ANOVA by statistical package (SPSS 12 for Windows). Post hoc test was carried out using Duncan's multiple range tests, if the treatments were significant.

3. Results

3.1. Expression of hsp 70

Induction of hsp 70 was evident in the muscle tissue of *C. carpio* at 36 °C in control groups (without chlorine). Interestingly, in the presence of 0.1 mg L^{-1} chlorine, induction of hsp 70 was inhibited, even at 36 °C (Fig. 1).

3.2. Haematological parameters

3.2.1. Haemoglobin content

Haemoglobin levels increased until 33 °C ($p < 0.05$) in temperature control groups (without chlorine). A general decreasing trend of haemoglobin levels was observed irrespective of the acclimation temperatures (in the presence of chlorine) ($p < 0.05$) (Table 1).

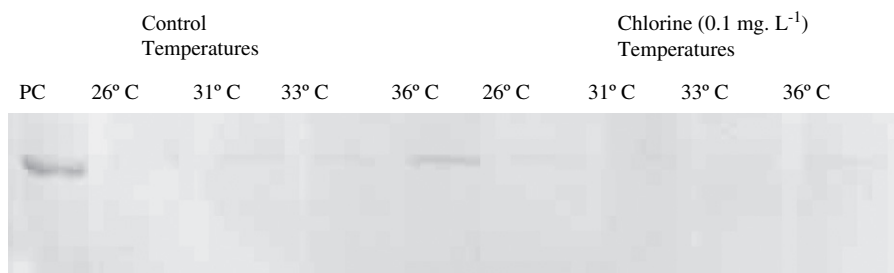


Fig. 1. Western blot showing induction of heat shock protein 70 in *C. carpio* muscle exposed to different temperatures in combination with chlorine. Lane 1 – positive control; lanes 2–5 (26, 31, 33 and 36 °C) control; lanes 6–9 (26, 31, 33 and 36 °C) + 0.1 mg L⁻¹ chlorine.

3.2.2. Total erythrocyte and leucocyte counts

Total erythrocyte levels increased in temperature until 33 °C ($p < 0.05$) in control groups (without chlorine). However, in the presence of chlorine, erythrocyte levels decreased significantly as compared to their respective temperature control groups ($p < 0.05$) (Table 1). A decrease in leucocyte count was observed with increasing temperatures (without chlorine) and a further decreasing trend was observed in the presence of chlorine ($p < 0.05$) (Table 1).

3.2.3. Serum protein, albumin, globulin and A/G ratio

Serum protein decreased with increasing temperatures in temperature control groups and a further decreasing trend was observed in the presence of chlorine ($p < 0.05$) except at 31 °C (Table 2). Albumin and globulin levels decreased with increasing temperatures. Significantly decreased levels of albumin and globulin were observed in the presence of chlorine at 36 °C (Table 2). However, A/G ratio was unaffected in chlorine treated groups over temperature control groups ($p > 0.05$). A significant ($p < 0.05$) decrease in A/G ratio was observed at 36 °C in both the groups irrespective of the chlorine treatment (Table 2).

3.3. Respiratory burst activity

The production of superoxide demonstrated a decreasing trend with increasing temperature in control groups ($p < 0.05$). A further augmented response was evident in chlorine treated groups at 26 and 33 °C (Table 3).

4. Discussion

Numerous studies have investigated the effect of temperature on the immunological responsiveness of ectothermal animals. In general, there is a direct effect of temperature on immunomodulation in fish. Increased intracellular levels of hsp 70 help the cell survive stresses by binding to partially denatured proteins and assisting to refold these proteins into more stable native structures. Therefore, acclimation to higher temperatures results in higher basal levels of hsp 70 [30]. In the present investigation, acclimation temperature dependent induction of hsp 70 was demonstrated at

Table 1
Effect of chlorine at four different acclimation temperatures on haemoglobin, total erythrocyte and total leucocyte counts of *C. carpio*

Parameter	Temperature (°C)				Temperature (°C) + 0.1 mg L ⁻¹ chlorine			
	26	31	33	36	26	31	33	36
Haemoglobin (gm/dl)	5.28 ± 0.17 ^c	5.51 ± 0.11 ^b	5.57 ± 0.29 ^a	5.50 ± 0.09 ^b	4.61 ± 0.12 ^{d*}	4.30 ± 0.11 ^{e*}	4.16 ± 0.25 ^{f*}	4.05 ± 0.12 ^{g*}
Total erythrocyte count (10 ⁵ cells/mm ³)	2.53 ± 0.03 ^d	2.60 ± 0.05 ^c	2.72 ± 0.03 ^b	2.90 ± 0.05 ^a	2.08 ± 0.03 ^{e*}	2.61 ± 0.11 ^c	1.70 ± 0.05 ^{f*}	1.60 ± 0.04 ^{g*}
Total leucocyte count (10 ⁴ cells/mm ³)	8.40 ± 0.02 ^a	7.46 ± 0.01 ^b	6.58 ± 0.1 ^c	5.83 ± 0.05 ^f	6.38 ± 0.2 ^{d*}	6.12 ± 0.6 ^{e*}	5.78 ± 0.9 ^{f*}	5.36 ± 0.3 ^{g*}

Values are expressed as mean ± SE ($n = 3$).

Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $\alpha = 0.05$). *Indicates the significance of chlorine treated groups over their respective controls.

Table 2
Effect of chlorine at four different acclimation temperatures on serum parameters of *C. carpio*

Parameter	Temperature (°C)				Temperature (°C) + 0.1 mg. L ⁻¹ Chlorine			
	26	31	33	36	26	31	33	36
Total serum protein (gm %)	5.78 ± 0.14 ^a	5.40 ± 0.06 ^c	4.82 ± 0.08 ^d	4.00 ± 0.04 ^e	5.68 ± 0.21 ^{b*}	5.44 ± 0.08 ^{bc}	4.32 ± 0.04 ^{e*}	2.92 ± 0.11 ^{f*}
Total albumin (gm %)	2.36 ± 0.07 ^a	2.21 ± 0.01 ^c	2.00 ± 0.02 ^d	1.26 ± 0.01 ^e	2.29 ± 0.01 ^{b*}	2.25 ± 0.02 ^{bc}	1.78 ± 0.02 ^{f*}	1.00 ± 0.01 ^{g*}
Total globulin (gm%)	3.41 ± 0.21 ^a	3.18 ± 0.06 ^{ab}	2.82 ± 0.08 ^{bc}	2.74 ± 0.03 ^c	3.47 ± 0.21 ^a	3.18 ± 0.08 ^{ab}	2.53 ± 0.04 ^c	1.91 ± 0.11 ^{d*}
A/G ratio	0.712 ± 0.063 ^b	0.696 ± 0.016 ^b	0.713 ± 0.028 ^b	0.460 ± 0.006 ^a	0.673 ± 0.036 ^b	0.710 ± 0.018 ^b	0.703 ± 0.013 ^b	0.533 ± 0.037 ^a

Values are expressed as mean ± SE ($n = 3$).

Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $\alpha = 0.05$). *Indicates the significance of chlorine treated groups over their respective controls.

36 °C (Fig. 1). However, persistent use of chlorine inhibited the induction of hsp 70 in *C. carpio* advanced fingerlings at thermal extremes (36 °C), which might be due to the inhibition of protein synthesis. Similar inhibition of protein synthesis was evident from our data on enzymes of protein metabolism; Alanine amino transferase and Aspartate amino transferase (unpublished data) when *C. carpio* advanced fingerlings were exposed to high temperatures in the presence of chlorine. Hsp 70 is an ATP-binding protein and has ATPase activity. The ATP-bound form of the protein has a low affinity for substrate, whereas the ADP-bound form can bind substrate. Therefore, regulation of the ATPase activity can affect the ability of hsp 70 to bind to and refold proteins [31]. Therefore, inhibition of hsp 70 in chlorine treated groups maintained at different acclimation temperatures may be due to lack of substrate (ATP) after 28 days of acclimation.

Haemoglobin (Hb) levels were increased with increasing acclimation temperatures in the control group, which might be due to the increase in oxygen demand. Similar haematological responses have been reported in *Clarias gariepinus* exposed to increasing temperatures [32]. Previous haematological studies of nutritional effects [33–36], infectious diseases [37] and pollutants [38] have demonstrated that erythrocytes are a major and reliable indicator of stress. From our study, it is evident that Hb concentration is significantly affected by persistent exposure to sub-lethal level of chlorine. RBC counts increased with increasing temperatures in the control group probably because of the increased demand for oxygen consumption. A similar trend was observed in *C. gariepinus* when exposed to increasing temperatures [32]. It is well known that a reduction in erythrocytes and a decrease in Hb levels may lead to a deteriorated oxygen supply. A significantly lower count was recorded at higher temperatures in the chlorine treated groups suggesting the inhibitory effect of chlorine affecting the production of erythrocytes, which is generally augmented at higher temperatures. Similar results were reported when Common carp, *C. carpio* L. were exposed to cyanobacteria extract [39]. An insufficient quantity and quality of red blood cells would therefore consequently have several additional effects on metabolism beyond the function of supplying oxygen for tissue metabolism [32].

Leukopenia is another haematological response considered pertinent [40]. Leucocyte count was higher in the control groups as compared to chlorine treated groups in our study, which corresponds to immunosuppression due to chlorine [41,42].

Table 3
Effect of chlorine at four different acclimation temperatures on respiratory burst activity in blood of *C. carpio*

Parameter	Temperature (°C)				Temperature (°C) + 0.1 mg L ⁻¹ chlorine			
	26	31	33	36	26	31	33	36
Respiratory burst activity (OD/540 nm)	0.303 ± 0.025 ^a	0.210 ± 0.023 ^b	0.205 ± 0.019 ^b	0.178 ± 0.013 ^{bc}	0.188 ± 0.015 ^{bc}	0.178 ± 0.012 ^{bc}	0.166 ± 0.013 ^c	0.153 ± 0.014 ^c

Values are expressed as mean ± SE ($n = 3$).

Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $\alpha = 0.05$). *Indicates the significance of chlorine treated groups over their respective controls.

Serum proteins are divided into two major groups, albumin and globulins. Serum globulin consists of several components of which three groups are easily identified as α -, β - and γ -globulin. The γ -globulin fraction is the source of almost all the immunoglobulins of blood [43]. Total serum protein was found to decrease with increasing temperatures in both the control as well as chlorine treatments. Similar decrease in serum protein levels was observed when *Labeo rohita* were exposed to bacterial challenge [44]. However, the effects of treatment did not show any clear effects on the A/G ratio in the present study in *C. carpio*.

It is generally accepted that fish phagocytes after activation are able to generate superoxide anion (O_2^-) [45,46]. The present results demonstrated a decreasing trend in superoxide production with increasing temperature and this decrease was exacerbated in the presence of chlorine. Interestingly, the findings of respiratory burst rate could be well correlated with the results of the leucocyte counts.

From our experiment, immunosuppression of *C. carpio* was evident at higher temperatures. A further augmented response was observed in chlorine treated groups over their respective temperature control groups. Another interesting observation is that chlorine exposure may affect the aerobic scope of the test fish. In accordance with Shelford's law of tolerance, decreasing whole animal aerobic scope characterizes the onset of thermal limitation at *pejus* thresholds (*pejus* means getting worse), as described by Portner in his review [47]. High temperature shifts the mitochondrial metabolism to anaerobic mode even in fully aerated water, which indicates a mismatch of oxygen supply and demand, which ultimately results in critically low tissue oxygen levels. Our results from haemoglobin and RBC levels indicate that oxygen carrying capacity was affected in chlorine treated groups. Prolonged stress beyond these limits may lead to progressive insufficiency of cellular energy levels in the test fish. Also, oxygen limitation contributes to oxidative stress and finally, denaturation or malfunction of molecular repair, due to suspension of protein synthesis [47]. In our study, a reduction in respiratory burst activity in chlorine treated groups further strengthens this hypothesis. Further, we speculate that failure to induce Hsp 70 at 36 °C in chlorine treated groups may be an indication of malfunction of molecular repair or protein denaturation.

Overall results indicate that exposure to high temperature increases immunosuppression in *C. carpio*. Presence of chlorine demonstrated a further augmented immunosuppressive response over their respective temperature control groups. Therefore, persistent chlorine exposure may affect the tolerance capacity of *C. carpio* to increasing temperature. To our knowledge, there are no parallel reports on immune response of chlorine and temperature in *C. carpio*. Therefore, this *prima-facie* laboratory based simulation study may invite attention of power plant operators to take adequate steps and ensure that thermal discharge from cooling towers do not accumulate chlorine at alarming levels. However, a field based analysis of chlorine levels and health status of fish at various points from discharge canal may further strengthen our simulation based hypothesis.

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