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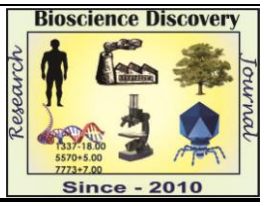
Research Article



In Vivo effects of cadmium chloride on antioxidant metabolism in sexually mature common carp *Cyprinus carpio*

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Abstract

Production of reactive oxygen species (ROS) and parameters of antioxidant defence mechanism in serum, ovary and liver of sexually mature *Cyprinus carpio* were studied after long-term $CdCl_2$ exposure. Production of ROS in serum, liver and ovary was elevated significantly in fish from day 1 to day 7 of $CdCl_2$ exposure with no further increase thereafter up to day 15 compared to their respective control values. Activities of total superoxide dismutase (SOD) and catalase in serum and ovary were gradually and significantly decreased from day 1 to day 4 followed by gradual increase for the rest of the exposure days. Liver SOD activity seemed to be distinctly responsive to $CdCl_2$. Results thus show that sub-lethal dose of $CdCl_2$ caused oxidative stress and antioxidant systems are very much effective to prevent damages caused by the generation of ROS.

INTRODUCTION

Heavy metal cadmium is a highly toxic environmental pollutant, discharged in large amount into the aquatic system by a number of industries, agricultural and mining activities. (Bhattacharyya *et al*., 2000; Singh *et al*., 2006; Sprocati *et al*., 2006). Chronic contamination of this heavy metal in the aquatic environment is a severe problem particularly to the living organisms. The situation is especially serious for its long time persistence in the environment with a half life of 15-30 years (Hensen and Anderson, 2000) and accumulation over times in blood, kidney and liver (Varga *et al*., 1993; Hensen and Anderson, 2000; Bhattacharyya *et al*., 2000), as well as in reproductive organs (Varga *et al*., 1993; Orlando *et al*., 2002). All these prompted numerous investigators to study the effects of this metal on the biological functions of fish, and in particular their defence mechanisms.

Fish are of potential interest as sensitive targets for addressing the environmental stress and/or pollution load, because they may be subject to an enhanced oxidative stress due to chronic exposure of pollutants (Kock *et al*., 1995; Whitefield and Elliott, 2002; Romeo *et al*., 2000). Cadmium may cause oxidative stress in aquatic organism by producing ROS (Seveikova *et al*., 2011; Wang *et al*., 2013). Oxidative stress occurs when ROS such as superoxide ion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet O_1 are produced. All these react with nucleic acids, protein and lipids resulting in several biochemical injuries (Pinchuk and Litchenberg, 2002; Valvanidis *et al*., 2006). One of the prerequisite of aerobic life is the detoxification of ROS (McCord, 2009), and many defences have been evolved providing an antioxidant system which is able to prevent, intercept and repair damages. These are non-enzymatic ROS scavenger and also enzymatic system such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, peroxidase, and glucose-6-phosphate dehydrogenase

(Sies, 1991; Valvanidis *et al*., 2006).

Hence, the responses or changes of these enzymes in fish tissues have been extensively used as an early warning bio-indicators particularly in relation to pollution of aquatic environment (Roche and Boge, 2000; Almeida *et al*., 2002; Klumpp *et al*., 2003; Valvanidis *et al*., 2006; Das *et al*., 2016). The aim of the present study is to evaluate the *in vivo* effect of $CdCl_2$ on antioxidant adaptive responses through determination of ROS generation and activity determination of SOD and catalase in long-term exposure of sexually mature female *C. carpio.*

MATERIALS AND METHODS

Chemicals

Cadmium chloride was purchased from Merck India (Cat. No. 61813101001730). SOD activity was assayed using SOD assay kit (Cayman Cat No. 706002). All other chemicals used were of analytical grade.

Animals

Adult female *C. carpio* at vitellogenic stage (400-500 g. body wt), collected from a local fish farm during the month of September to October were kept in outdoor concrete tanks (300 L capacity) at 23 ± 1^0C at least for 5 days prior to experiment. They were fed with commercial fish food (Shalimar fish food, Bird and Fish food manufacturer, Mumbai). Follicular developmental stage was determined according to the procedure described previously (Paul *et al*., 2008; 2010). During the month of September to October, ovaries of female carp in the plains of West Bengal, India comprise mostly vitellogenic follicles (0.3-0.4 mm in diameter) with oocyte containing centrally located germinal vesicle. The cytoplasm was filled with yolk granules and cortical granules were shown to cover the entire oocytes.

In vivo exposure of fish

Toxicity bioassay of $CdCl_2$ was conducted according to the standard procedure outlined by Doudoroff *et al*. (1951). Altogether 25 healthy vitellogenic fish were exposed to the sub-lethal concentration of $CdCl_2$ (10% of TLM conc. of $CdCl_2$ i.e., 0.65mg /L) for 1, 2, 4 and 7 and 15 days. A parallel control was run simultaneously. Both control and test solution was renewed daily, and sampling of fish was done after 1, 2, 4 and 7 and 15 days of exposure respectively. After sampling, fish were bled for the separation of serum and killed by decapitation. Ovaries and liver from each fish were taken out, washed in PBS and minced under ice in aseptic condition. Minced ovary and liver tissues and serum were kept at −80°C for the estimation of ROS, SOD and catalase activity.

Determination of reactive oxygen species (ROS)

Determination of ROS in liver, serum and ovary was done by quantifying hydrogen peroxide and hydroxyl radical production following the procedure described by Murugesan *et a*l. (2000**)**, and as reported previously (Das *et al*., 2016). Hydrogen peroxide was quantified following the method of Holland and Storey (1981) and as reported previously (Das *et al*., 2016). The H_2O_2 and hydroxyl radical content of the sample was expressed as µmol/min/mg protein.

Estimation of superoxide dismutase (SOD) enzyme activity

Superoxide dismutase (SOD) enzyme activity was estimated following the Cayman SOD assay kit instructions as described previously (Das *et al*., 2016). Minced liver and ovarian tissues were homogenized in 5 ml cold 20 mM HEPES, 1mM EGTA, 210 mM mannitol and 70 mM sucrose. The homogenate was then centrifuged at 1,500g for 5 min at 4ºC and supernatant was collected and stored at −80ºC for SOD assay. Serum was diluted with sample buffer in 1:5 ratios. The SOD activity of the sample was expressed as U/gm tissue protein or ml of serum. The SOD activity of samples was calculated following the instructions of assay kit. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The intra-assay and inter-assay coefficient of variations of the method was 3.2 and 3.7% respectively.

Estimation of catalase activity

Liver and ovarian tissue was homogenized under ice with 10 mM Tris- buffer (pH-7.4) containing 1mM EDTA and 70 mM sucrose. The homogenate was centrifuged at 15,000 g at 4^0C for 20 min (Lijun *et al*., 2005). Catalase activity was determined following the procedure of Beer and Sizer (1952), as used in our laboratory (Das *et al*., 2016). Catalase activity was calculated and expressed as mM H_2O_2 metabolized/ mg tissue protein or/ ml serum.

Statistical analysis

Data obtained from three replicate incubations of all the tissues from a single donor fish showed similar tendency and mean of them was considered as one experiment. All data were expressed as mean ± S.E.M. of five such experiments taking samples from five donor fish. Following test for normality and homogeneity, the significance of treatment

effect was determined by one-way ANOVA followed by Bonferroni's multiple comparison test using SPSS (Chicago, IL, USA) with a significance level $p < 0.01$ and $p < 0.05$.

RESULTS

Effect of cadmium chloride on reactive oxygen species (ROS)

The levels of ROS generation such as hydrogen peroxide and hydroxyl radical in control and $CdCl_2$ exposed fish are shown in Fig. 1 and 2 respectively. Exposure of fish to sub-lethal concentration of $CdCl_2$ for 15 days gradually and significantly ($p<0.01$) elevated hydrogen peroxide (Fig. 1) and hydroxyl radical **(**Fig. 2**)** generation in serum (A), liver (B) and ovary (C) up to day 7 of exposure compared to their respective control values. However, no further increase of both hydrogen peroxide and hydroxyl radical in all the tissues was recorded from day 7 to day 15 exposure of $CdCl_2$ compared to day 7 exposure.

Effect of cadmium chloride on ovarian SOD activity

Fish exposed to sub-lethal concentration of $CdCl_2$ caused gradual and significant ($p<0.01$) attenuation in serum and ovarian SOD activities from day 2 of exposure compared to their respective control values and this decrease was continued with a maximum on day 7 in serum and day 4 in ovary (Fig. 3A and C). SOD activities thereafter increased significantly up to day 15. The liver SOD activity seemed to be distinctly responsive to $CdCl_2$. SOD activity in liver was started to increase after day 1 exposure and continued gradually and significantly ($p<0.05$) up to day 4 with no further increase thereafter (Fig. 3B).

Effect of cadmium chloride on catalase activity

Fig. 4 shows that serum, liver and ovarian catalase activities were gradually inhibited from day 1 of $CdCl_2$ exposure and significant inhibition ($p < 0.01$) was noticed up to day 4 in liver and day 7 in serum and ovary **(**Fig. 4A-C) compared to their respective control values, Catalase activity, thereafter increased significantly ($p<0.01$) in all the three tissues up to 15 day of exposure.

DISCUSSION

The present study showed that *in vivo* exposure of $CdCl_2$ for 15 days caused elevated ROS production in ovary, liver and serum of this fish. Ovarian and serum SOD activities, however, after an initial decrease, increased significantly with increasing days and maximum was recorded at day 15, whereas liver SOD activity started to increase from day 1 of exposure with a maximum at day 7 with no further increase thereafter. Catalase activity of all the tissues tested also decreased up to day 4 or day 7 of $CdCl_2$ exposure and increased thereafter up to day 15.

In the present study, there was an elevated level of ROS (H_2O_2 and OH^- radicals) in ovary, liver and serum after $CdCl_2$ exposure of fish. Oxidative damage occurs when ROS react with cellular lipids, proteins and nucleic acids (Wiseman *et al*., 1996; Roberts *et al*., 2009**)**. $CdCl_2$ has also been reported to increase ROS generation in tissues and inhibits the activity of some enzymes of the antioxidative defense system (Jackim *et al*., 1970; Pruell and Engelhardt, 1980; Zikic *et al*., 1996). ROS can damage critical components of the steroidogenic pathway in Leydig cells, including StAR protein (Diemer *et al*., 2003) and cytochrome P450 enzymes. Available information although indicated the higher production of ROS and its deleterious effects on several steroidogenic enzymes (Murugesan *et al*., 2007). The present study showed for the first time that $CdCl_2$ can increase the ROS production in fish ovary.

It has also been observed that an increase in free radical formation results in the inhibition of antioxidant defence system during exposure to $CdCl_2$. The antioxidant metabolism of *C. carpio* was responsive to environmental presence of $CdCl_2$. Ovary of *C. carpio* presented alteration in the enzyme activity after exposure to $CdCl_2$. SOD activity in fish tissues have been extensively used as an early warning bio indicator particularly in relation to pollution of aquatic environments (Almeida *et al*., 2002; Klumpp *et al*., 2002; Basha and Rani, 2003; Pandey *et al*., 2003). SOD catalyzes the reduction of superoxide anion to H_2O_2, and it is expected that fish exposed to oxidative stress will changes the activity of this enzyme. Decreased SOD activity were reported in cadmium exposed *Carassius auratus gibelio* and carp (Zikic *et al*., 1997; 2001). Present data in common carp shows that activities of SOD in serum and ovary decreased gradually from day 1 to day 4 exposure compared to their respective control values. However, after the day 4, activities of this enzyme increased gradually and significantly up to day 15. These results indicate that immediately after exposure to toxicant, fish was not able to activate its protective mechanism necessary for scavenging of produced O_2^- radical in serum and ovarian follicles. But prolonged exposure up to day 15 caused

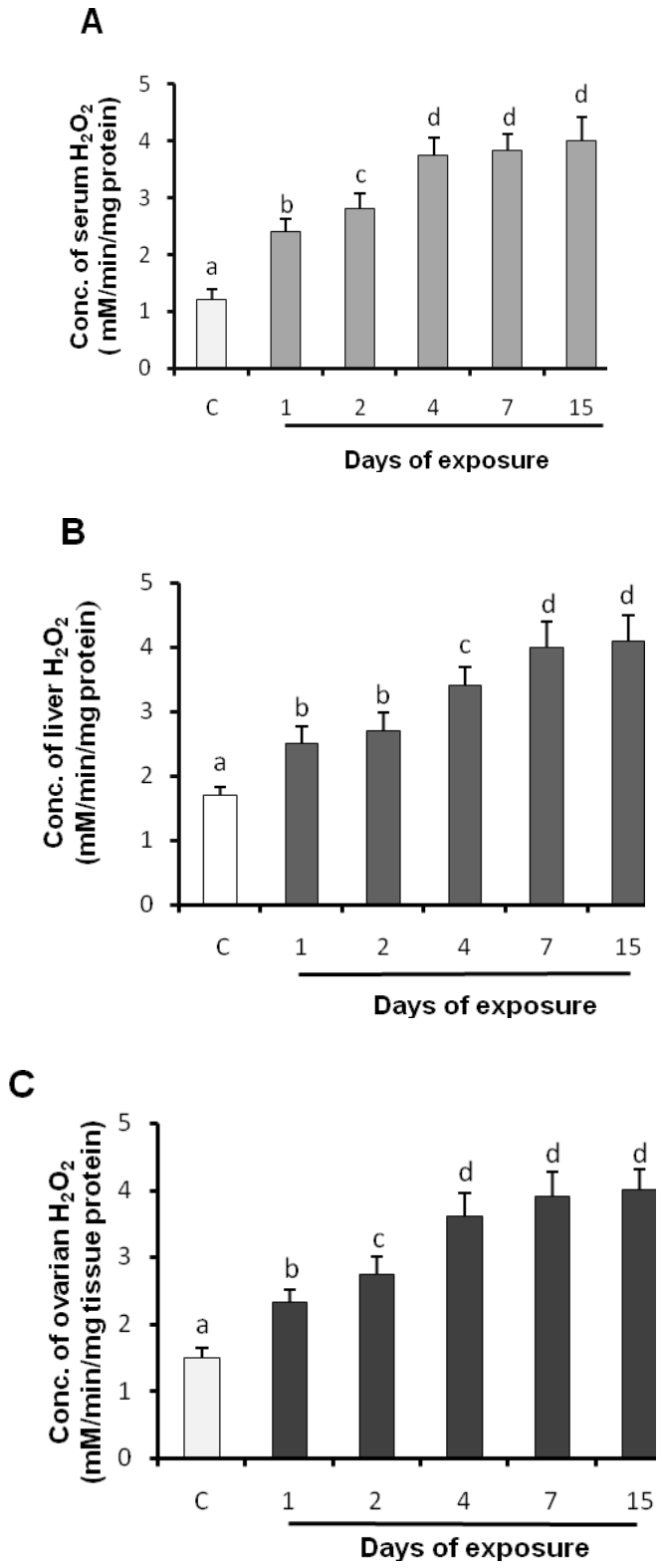

Fig.1: Time-course effect of cadmium chloride (0.65 mg/L) on *in vivo* production of hydrogen peroxide (H_2O_2) in serum (**A**), liver (**B**) and ovary (**C**) of *C. carpio*. Fish were exposed to toxicant for 1, 2, 4, 7 and 15 days. Details of experiments have been described in "Materials and methods" section. Experiments were performed five times in triplicate taking tissues from five donor fish and values are mean ± SEM. Bar associated with different letters are significantly ($p<0.01$) different. C- Control.

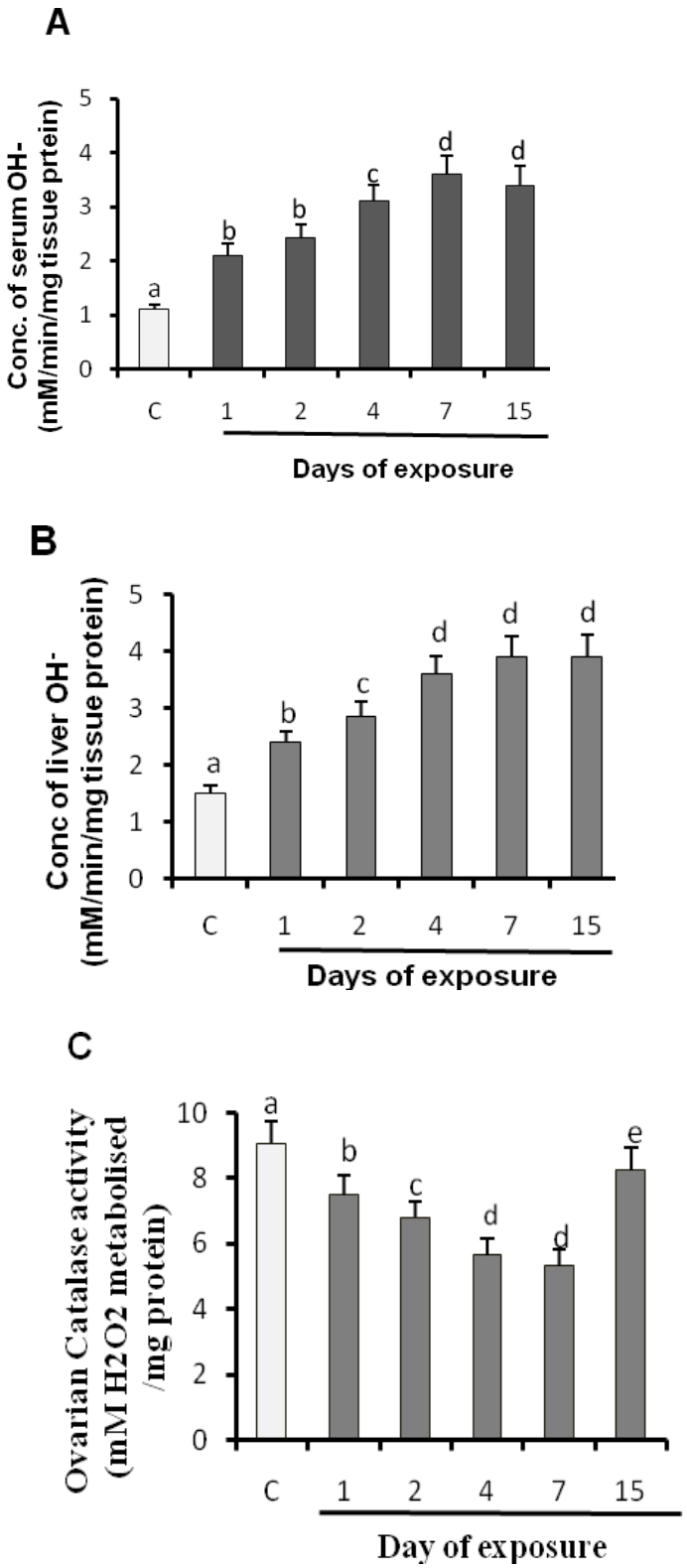

Fig. 2: Time-course effects of cadmium chloride (0.65 mg/L) on *in vivo* production of hydroxyl radical in serum (**A**), liver (**B**) and ovary (**C**) of *C. carpio*. Fish were exposed to toxicant for 1, 2, 4, 7 and 15 days. Details of experiments have been described in "Materials and methods" section. Experiments were performed five times in triplicate taking tissues from five donor fish and values are mean ± SEM. Bar associated with different letters are significantly ($p<0.01$) different. C- Control.

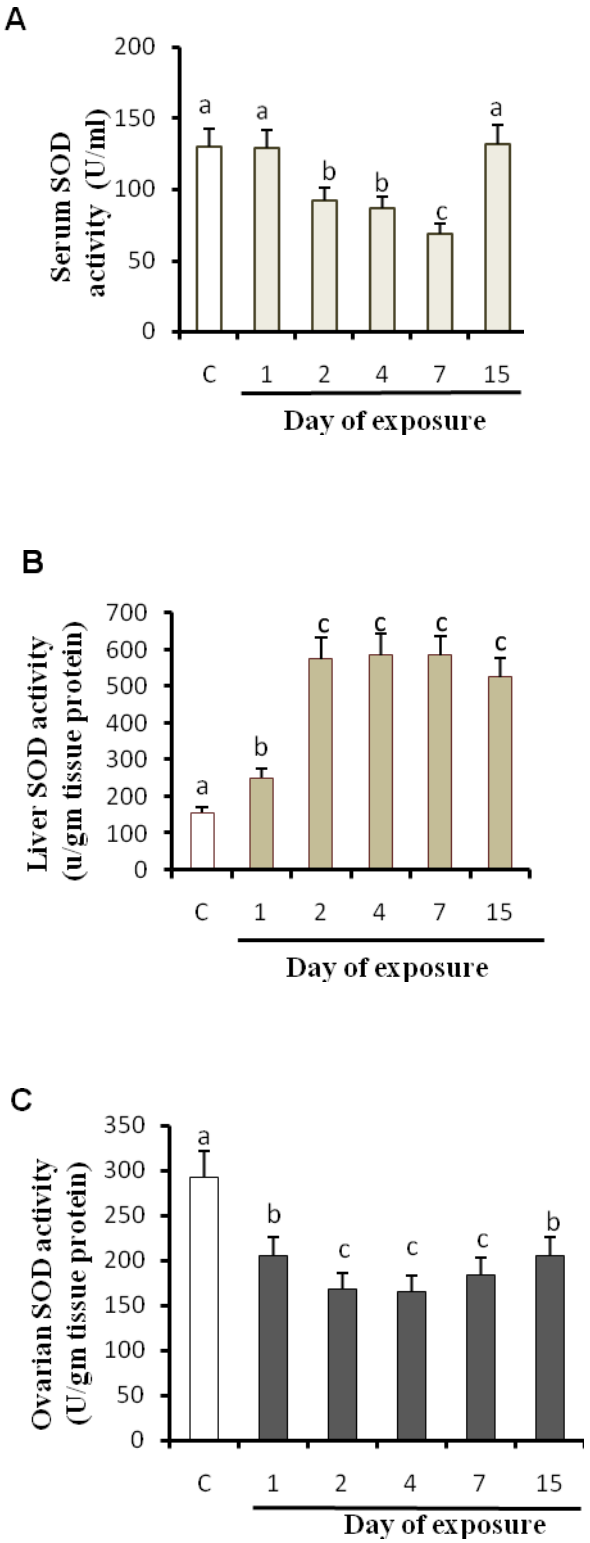

Fig. 3: Effect of cadmium chloride (0.65 mg/L) on SOD activities of serum (**A**), liver (**B**) and ovary (**C**) of vitellogenic-stage common carp. Fish were exposed to toxicant for 1, 2, 4, 7 and 15 days. Details of experiments have been described in "Materials and methods" section. . Experiments were performed five times in triplicate taking tissues from five donor fish and values are mean ± SEM. Bar associated with different letters are significantly ($p<0.01$) different. C- Control.

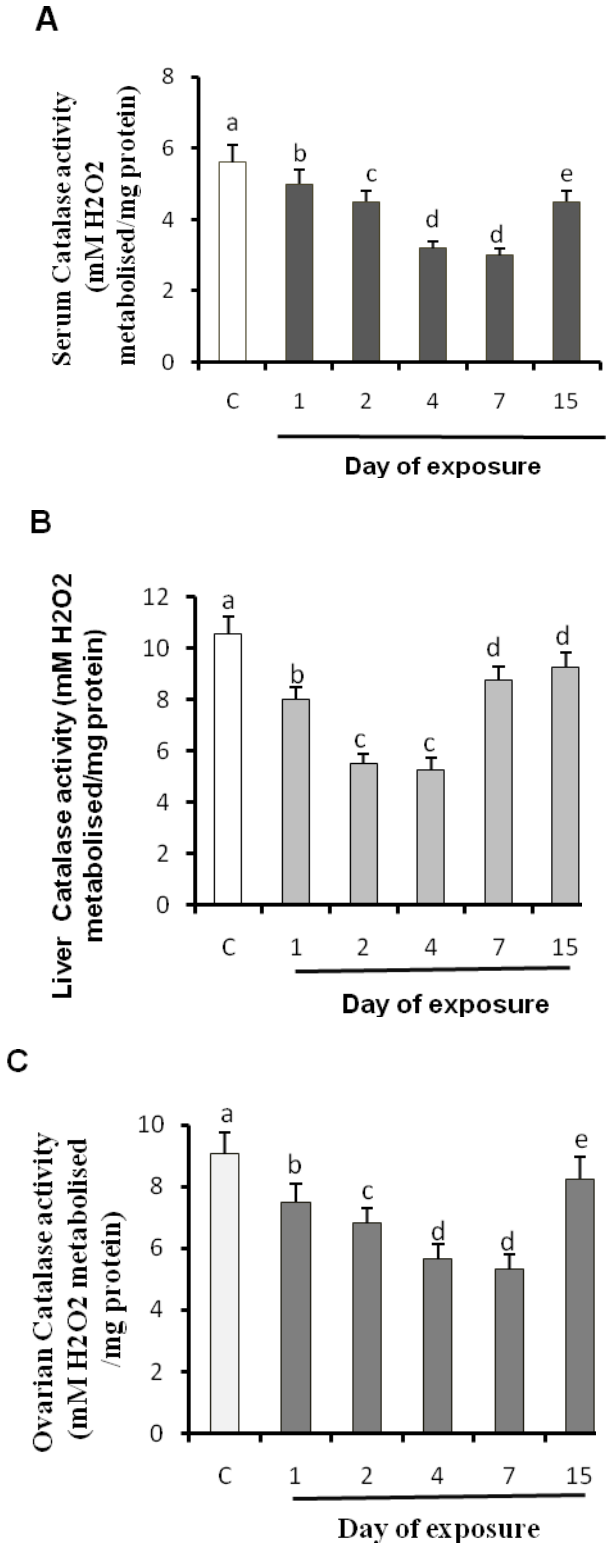

Fig. 4: Effect of cadmium chloride (0.65 mg/L) on catalase activities in serum (**A**), liver (**B**) and ovary (**C**) of vitellogenic-stage common carp. Fish were exposed to cadmium chloride for 1, 2, 4, 7 and 15 days. Details of experiments have been described in "Materials and methods" section. Experiments were performed five times in triplicate taking tissues from five donor fish and values are mean ± SEM. Bar associated with different letters are significantly ($p<0.01$) different. C- Control.

Fish were exposed to toxicant for 1, 2, 4, 7 and 15 days. Details of experiments have been described in "Materials and methods" section. Experiments were activation of such protective mechanism. Previous studies also have shown that prolonged exposure to cadmium may enhance the activity of this enzyme in some mammalian tissues such as intrascapular brown adipose tissue (Kostic *et al*., 1993) and also in gold fish (Zikic *et al*., 2001). Results of the present study demonstrate that activities of SOD in liver increased immediately after exposure of fish to cadmium and this increase was maximal up to day 4 of exposure, after which there was no further change. These results indicate that cadmium in fish liver primarily act as oxidant or producer of free radical. Immediate increase of the activity of this enzyme shows a good protective mechanism necessary for scavenging O_2^- radical in the liver of this fish. Earlier studies with many animals showed that SOD activity varies with varying concentration of cadmium. The increased SOD activity in the liver of *C. carpio* may be explained as a compensatory mechanism against heavy metal intoxication which was similar to the result observed with increase SOD activity in carp after exposure to zinc and lead (Dimitrova *et al*., 1994; Farombi *et.al*., 2007).

Catalase activity is fundamental to remove hydrogen peroxide from the cytoplasm. It has been shown that H_2O_2 was produced and metabolized by catalase and its activity is directly regulated by the concentration of H_2O_2 (Fornazier, 2002). The peroxy radical of H_2O_2 was trapped by catalase in peroxisomes. The target function of catalase is to protect the cells from the accumulation of H_2O_2 by dismuting it to H_2O and O_2 or by using it as an antioxidant where it works as peroxidase. Decreased activity of catalase in ovary after exposure to $CdCl_2$ shows that immediately after the exposure fish was not able to protect the cells from the accumulation of H_2O_2. Pruell and Engelhardt (1980) by studying cadmium poisoning in the killifish, *Fundulus heteroclitus*, found that catalase activity was inhibited following both in *vivo* and *in vitro* exposure to dissolved cadmium at a concentration greater than 1 mg l^{-1}. Once the fish was exposed to 1 or 10 mg l^{-1} Cd, further *in vitro* addition did not change liver catalase activity. Pre-exposure may cause the formation of a detoxifying system which then limits the inhibitory effect of cadmium when added *in vitro.* The authors suggested a direct effect of cadmium on high molecular weight compounds like discernible effect in catalase activity of the liver initially but after 8 days of exposure, the activity increased in liver and thereafter, decreased gradually (Rajamanickam and Narayanan, 2009). Similar mode of catalase activity has also been reported in cadmium treated *Oxya chinensis* (Lijun *et al*., 2005). An inhibition of catalase in liver of rainbow trout exposed to cadmium was reported by Palace *et al*. (1992) who estimated that possible mechanisms by which cadmium produces lower catalase activity, may include direct metal-mediated structural alteration of the enzyme and depression of catalase synthesis. Similar mode of catalase activity in the blood of Sea bass (Roche and Boge, 1996) and in the blood of Matrinxa exposed to cadmium chloride has been reported (Avilez *et al*., 2008). Catalase inhibition by O_2^- was previously reported by Kono and Feridovich (1982). In the present study decreased catalase activity may be attributed to ineffective scavenging of H_2O_2 leading to increased H_2O_2 as observed in $CdCl_2$ exposed *C. carpio*.

Present study indicates that cadmium chloride toxicity results increased generation of ROS. Furthermore, for detoxification of the toxicant, good defence systems have evolved which are able to prevent, intercept and repair damages in this fish species.

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