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RESEARCH ARTICLE

ULTRA STRUCTURAL AND BIOCHEMICAL CHANGES INDUCED BY CADMIUM NANOPARTICLE IN THE MUSCLE OF FRESH WATER FIDDLER CRAB, *SCYLLA OLIVACEA* (HERBST, 1796) FROM PULICATE LAKE, TAMIL NADU

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ABSTRACT

Nanotechnology is an emerging technology which deals with the production and application of Nanoparticles of heavy meals in various fields of day to day life such as food, cosmetics, medicine, pharmacy, energy, environment and material application. But its industrial runoff may affect the aquatic ecosystem. In the present study ultra structural and biochemical changes induced by Cadmium nanoparticle in the Muscle of fresh water fiddler crab, Scylla olivacea (HERBST, 1796) was studied. Histological results revealed the loss of muscle structure, necrosis and atrophy of the muscle. Antioxidant enzyme analysis showed decreased activity of mitochondrial enzymes which re ect the disturbances of oxidation–reduction processes taking place in this organelle. The decreased activities in GPx and CAT, along with the increased activity in SOD, suggesting that there must be an oxidative stress in response to acute intoxication of Cd and that an oxidative damage was induced by the histopathological damage at the CdNP concentrations (20 ppm/kg body weight). Imbalance in the metabolic enzymes may decline the level energy in the organ system and in response to cadmium exposure the increase in LDH activity and declined SDH, MDH activity may help to maintain a balance in the energy requirement of the organism during the exposure to sublethal concentration of cadmium nano particles. So the overall results revealed the heavy metals in nanoscale may affect the ecobalance in the aquatic system which is reflected in the decrease in the population of the aquatic animals exposed the heavy metals intoxication.

INTRODUCTION

Marine pollution constitutes an ever-growing problem, especially in estuarine and coastal areas adjacent to densely-populated and industrialized regions. Adverse environmental effects associated with redistribution of trace metals due to mining and fossil fuel combustion have long been recognized (Leland and Kuwabara 1985 and Wren *et al.,* 1995). The fact that metal pollutants are persistent in the environment makes these contaminants especially hazardous (Viarengo, 1989). Cadmium (Cd) is one of the toxic heavy metals exposed from smoking, air, and food and water contaminated by Cd due to anthropogenic pollution (Nagata *et al.,* 2005, Ivanina *et al.,* 2008, 2010; Sokolova *et al.,* 2004). It can be accumulated in aquatic animals (e.g. crabs, shrimps, oysters and mussels) through respiratory tract, digestive tract or through surface penetration (Dailianis *et al.,* 2009; Ivanina *et al.,* 2008, 2010) and affecting the survival of the animals and their balance in the ecosystem. As aquatic food the animals exposed to cadmium also affect the human health. In recent days the nanotechnology is an emerging technology which deals with the production and application of Nanoparticles of heavy meals in various fields of day to day life such as food, cosmetics, medicine, pharmacy, energy, environmental, catalytic and material application and so on (Maynard, 2006; Farre *et al*., 2009, Fabrega *et al.,* 2011a). But it has negative impact on the environment and those Nanoparticles from industrial sources will affect the population of the animals belongs to that environment (Service, 2008; Nel *et al.,* 2006, Kahru and Dubourguier, 2010). While the behavior of NP in the environment (Biswas and Wu, 2005; Wiesner *et al*., 2006) and their ecotoxicology (Colvin, 2003; Moore, 2006) have been less often studied. However, no systematic description of the effects of NP on living organisms is yet to be investigated. Hence, the present study was designed to investigate the

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biochemical and histological changes in the muscle of *Scylla olivacea* after CdNP exposure.

MATERIALS AND METHODS

Animal Collection

Fresh samples of *Scylla olivacea* i.e, both male and female species are collected from Pulicate Lake, Tamil Nadu, India. They are maintained separately in tanks with aerator which is (capacity of 1000 litres) filled with filtered sea water. The sea water is changed periodically and crabs are fed with commercial fish feed. The morphological identification and authentication of species is done by a Scientist from Central Institute of Brackish water Aquaculture (CIBA), Santhome, Chennai, India. The crab is acclimatized for further studies for ten days before start of the experiment. During the acclimatization period, the specimens are fed twice a day. Naturally aged estuarine water is used after being shifted through a 0.45 mm pore filter and activated charcoal to remove dissolved organic matter and trace metals. Water temperature is maintained within a range (27.5± 0.5°C) as recommended for optimal growth of mud crabs (Chen and Jeng, 1980).

Acute toxicity test

The acute semistatic toxicity test was carried out according to the standard methodology for this kind of study such as those of the *Food and Agriculture Organization* (FAO) (Ward and Parrish, 1982; Reish and Oshida, 1987) and the American Public Health Association (APHA, 1992). Young crabs are acclimatized for 14 days. The size at the onset of sexual maturity in *S. olivacea* is considered as a young crab entails (17 mm carapace width for females and a 10 mm carapace width for males). Semistatic toxicological bioassays are carried out for 120 hrs. A series of six different concentrations such as 20, 40, 60, 80, 100 and 120 ppm of Cadmium nanoparticle (CdNP) suspension of 100nm in size (Sigma and Co) was injected intraperitonially per kg of crab weight. Three replicates of 10 animals are exposed to the above-stated concentrations. The criteria to determine death is the complete absence of movement once the animals were gently touched with a glass rod. Mortality is recorded every 24 h, a period of time after which dead crabs are removed. The experimental conditions (temperature, salinity, and pH) of the toxicity test are similar to those found in the environment during the period. To match the environmental conditions, an average of these parameters is used. A probit analysis is used to estimate the concentration and 95% confidence limits of SNP that kills 50% of the exposed crabs (LD_{50}).

Cadmium nanoparticle treatment

After, standardization of LD_{50} value, a single concentration of 20ppm/kg of body weight is used for further experiments. Mud crab, *S. olivacea i*s acclimatized in tanks and the temperature is maintained at 27°C. Water is changed daily and aquaria are cleaned thoroughly, and crab are fed with commercial fish feed. After acclimatization, healthy adult male and female crabs with a homogeneous size (carapace width 14-16cm, weight 200-300g) are selected for control and Cadmium nanoparticle (20 ppm/kg of crab weight) treatment. The acute exposure lasted for 8 days. During the experiment, crabs are fed and dead animals were removed in time.

Light Microscopic analysis

Cadmium nanoparticle treated and control crabs of both male and female *S. olivacea* are taken from the tank, anaesthetized in ice water for five minutes and sacrificed at every 2 day interval up to 8 days. Morphological changes of crab tissues in both control and CdNP treated groups are recorded and documented. Muscle tissues are removed and then fixed by direct immersion in a 0.1 M, pH 7.4 phosphate buffer with 4% formaldehyde for 24 h at room temperature. Samples are dehydrated with ethanol and toluene series and embedded in paraffin. Serial sections (4 mm) are mounted on gelatin-coated glass slides and stained with hematoxylin and eosin. Slides are examined with a light microscope (Olympus BX51) and the results are documented. Four sections were analyzed from each tissue.

Biochemical Analysis

Total Protein, Carbohydrate and Lipid analysis: The buffer soluble protein content of muscle tissue is determined by the dye binding method of Bradford (1976) with Bovine Serum Albumin fraction V (Sigma chemical Co., USA) as a standard. The gravimetric, chloroform-methanol extraction method of Folch *et al.,* (1957) was followed for lipid estimation in the muscle tissue. Total carbohydrate was estimated by Roe (1955). 10 % homogenate of tissues was prepared using 5 % TCA and results are expressed as mg/g tissue wet weight.

Antioxidant enzymes analysis: The antioxidant enzymes such as catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) is estimated in the muscle to determine the free radical formation in the tissues. Antioxidant activity of muscle tissues of *S. olivacea* after exposed to CdNP exposure was assayed colorimetrically. The Assays of Catalase, Superoxide Dismutase (SOD) is performed by following the procedures of Beers and Sizer (1952), Beyer and Fridovich (1987) and Glutathione Peroxidase by Lawrence and Burk (1976).

Tissue damaging enzymes analysis: After the CdNP exposure the tissue damaging enzymes in muscle tissues are colorimetrically assayed. The procedure of Schirawski and Unden **(**1998) is followed for Succinate Dehydrogenase (SDH), Gloster and Harris (1962) procedure for Lactate Dehydrogenase (LDH) and Bergmeyer (1983) procedure for Malate Dehydrogenase (MDH) is followed for the estimation of the enzymes.

Statistical analysis

Experiments were carried out in three duplicates. The data were tabulated and analyzed and Significant Difference (SD) analysis was performed to group the treatment mean values.

RESULTS

Histological changes in the muscles of male crab, *S. olivacea* : Muscle tissue of the control crab was made up of muscle cells containing contractile laments that move each other and change the size of the cell. Muscle tissue derived from mesoderm contains protein, and myosin lament (thread-like) form multi nucleate cells that assemble into bers called myo brils (Fig.1A). On day 2, the changes are necrosis and

appearance of granular material in between the muscle bers (Fig.1B). On day 4, atrophy and wavy appearance of the muscle bers, fragmentation of the muscle bers and intermuscluar areas with granular exudates are observed (Fig.2B). On day 6, wavy appearance of basophilic deposits, and atrophy and focal disappearance of the muscle bers are marked (Fig. 3B). On day 8, muscle tissue derived from mesoderm contained protein, and myosin filaments (thread-like) formed multi nucleate cells that assembled into fibers called myofibrils, atrophy and necrosis, loss of muscle structure and necrosis are observed (Fig.4B).

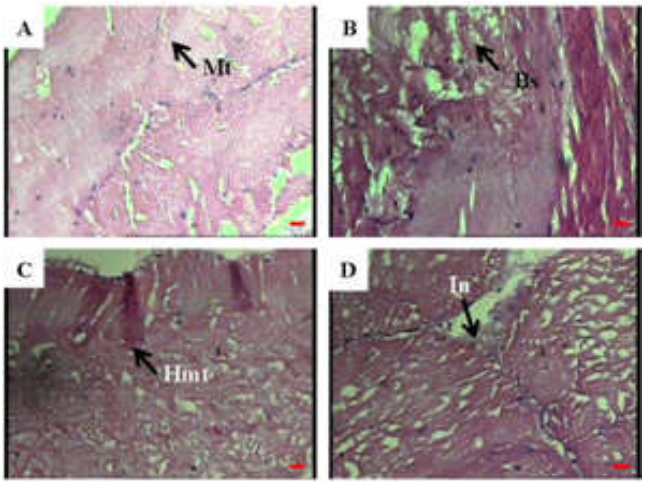

Figure 1. Histological changes in the crab, *S. olivacea* after exposure of CdNP on the 2nd day

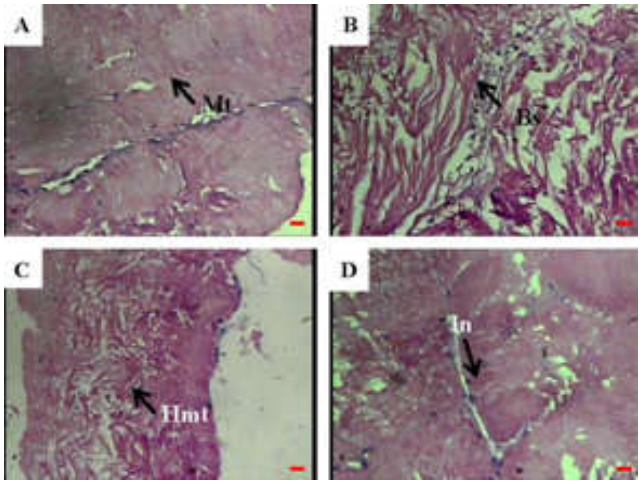

Figure 2. Histological changes in the crab, *S. olivacea* after exposure of CdNP on the 4nd day

Histological changes of muscles of female crab, *S. olivacea*

In female *S.olivacea*, control crabs showed ordered structure of contractile laments (Fig.1C). On day 2, the changes were slight necrosis followed by appearance of granular material in between the muscle bers (Fig.1D). On day 4, disorganisation of the muscle bers and fragmentation of the muscle bers (Fig.2D) were observed. On day 6, absence of wavy appearance of basophilic deposits, but focal disappearance of the muscle bers were marked (Fig.3D). On day 8, multi nucleate cells that assembled into fibers were evident, complete loss of muscle structure and basophilic granules were visible (Fig.4D).

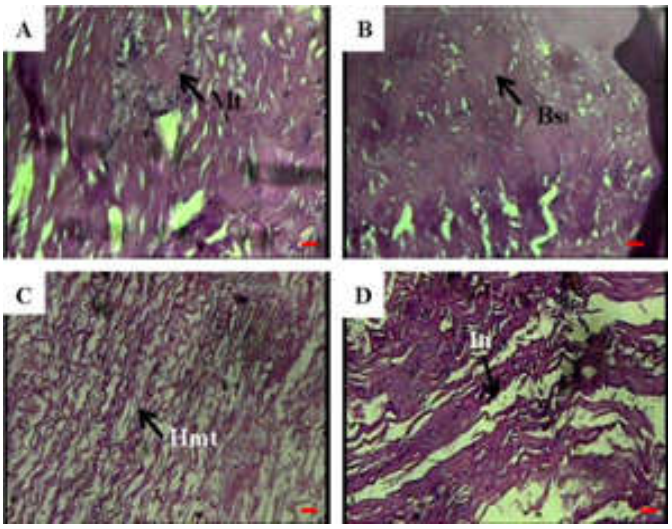

Figure 3. Histological changes in the crab, *S. olivacea* after exposure of CdNP on the 6nd day

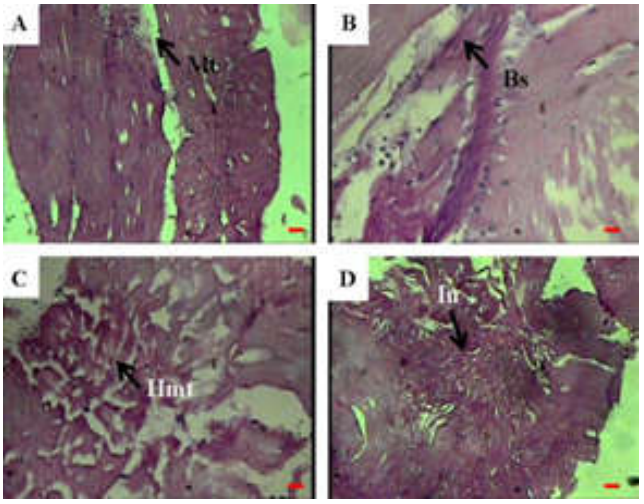

Figure 4. Histological changes in the crab, *S. olivacea* after exposure of CdNP on the 8nd day

Effects of CdNP on Total Protein Content of Muscles

A time course study of muscles of *S. olivacea* showed an increase in the total protein content upon exposure to CdNP. Total Protein content started increasing in CdNP treated male crabs on day 2 and reached peak on day 10. Similarly, in the case of female also total protein content started increasing on day 2 and reached peak on day 10 of exposure (Figure 5).

Effects of CdNP on Total Carbohydrate Content of Muscles

Results of total carbohydrate content in the muscles of *S. olivacea* exposed to Cadmium nanoparticle (20ppm) were presented in Figure 6. In general, CdNP resulted in decreased total carbohydrate content than in control crabs. In males, total carbohydrate content started decreasing on day 2 and reached lowest on day 10 of exposure. Similarly, in the case of female also carbohydrate content started decreasing in exposed crabs right on day 2 and reached maximum increased on day 10 of exposure.

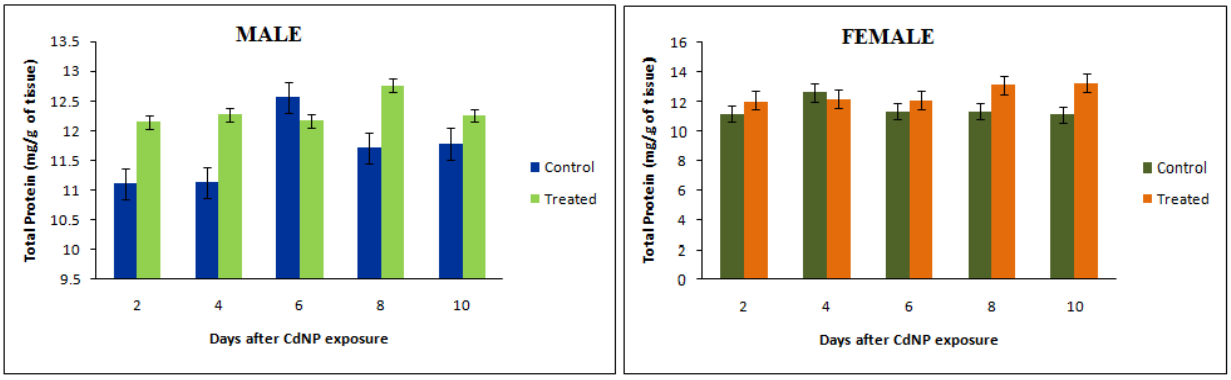

Figure 5. Total protein content in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

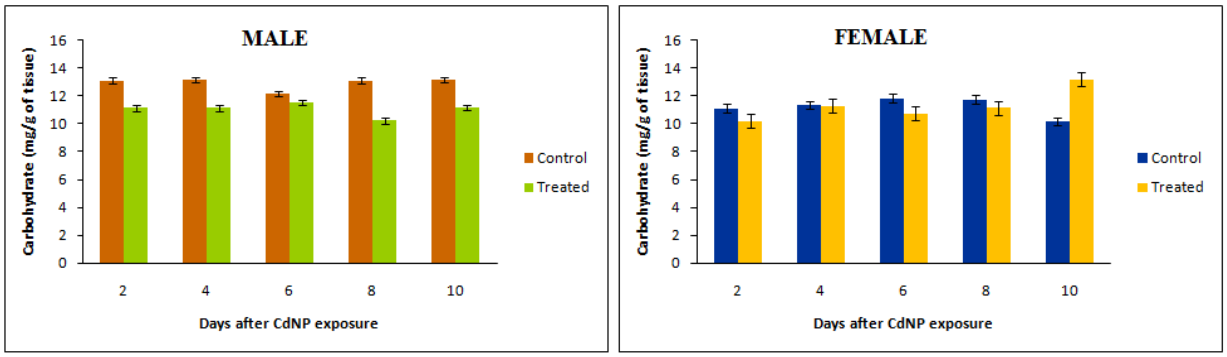

Figure 6. Total Carbohydrate content in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

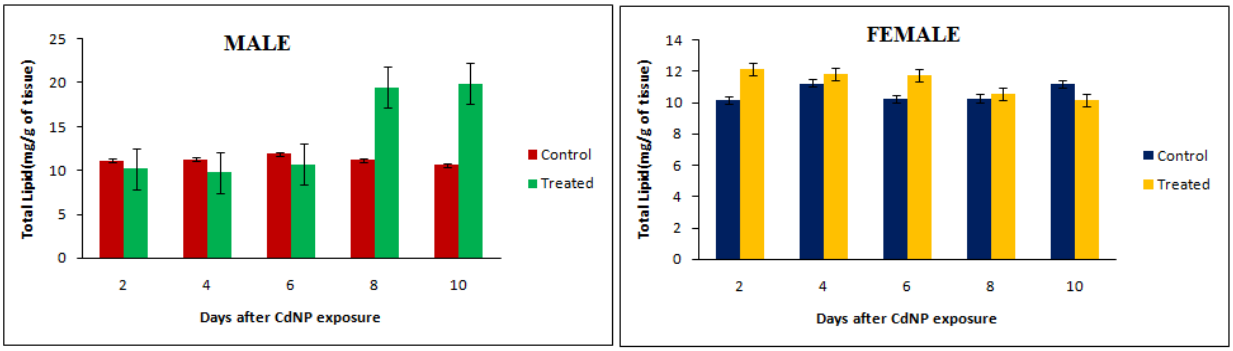

Figure 7. Total Lipid content in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

Effects of CdNP on Total Lipid Content of Muscles

The muscles of *S. olivacea* showed a decrease in total lipid content upon exposure to CdNP. Total lipid content started decreasing in CdNP treated male crabs on day 2 and reached increased on day 10(Figure 7). Similarly, in the case of female also total lipid content started increasing on day 2 and decreased on day 10 of exposure compared to their control.

Effects of CdNP on Catalase (CAT) activity

Cadmium nanoparticle (20ppm) exposure resulted in increased Catalase (CAT) activity in the muscles of *S. olivacea* than in control crabs. In male, catalase activity started increasing on day 2 and reached peak on day 10 of exposure (Figure 8). Similarly, in the case of female also catalase activity started increasing on day 2 and reached peak on day 10 of exposure. A maximum of upto six fold increments in the CAT activity was recorded in both male and female crabs compared to control.

Effects of CdNP on Superoxide Dismutase (SOD) activity

Cadmium nanoparticle exposure resulted in increased in SOD activity in the muscles of *S. olivacea* than in control crabs. In males, SOD activity started increasing on day 2 and reached peak on day 10 of exposure (Figure 9). Similarly, in the case of female also SOD activity started increasing on day 2 and reached peak on day 10 of exposure. A maximum of upto two fold increases in SOD activity was recorded in male and female crabs respectively compared to their respective control.

Effects of CdNP on Glutathione Peroxidase (GPx) activity

Cadmium nanoparticle exposure resulted in increased Glutathione Peroxidase (GPx) activity in the muscles of *S. olivacea* than in control crabs. In male crabs, GPx activity started increasing on day 2 and reached peak on day 10 of exposure (Figure 10). Similarly, in the case of female also GPx activity started increasing on day 2 and reached peak on day 10 of exposure.

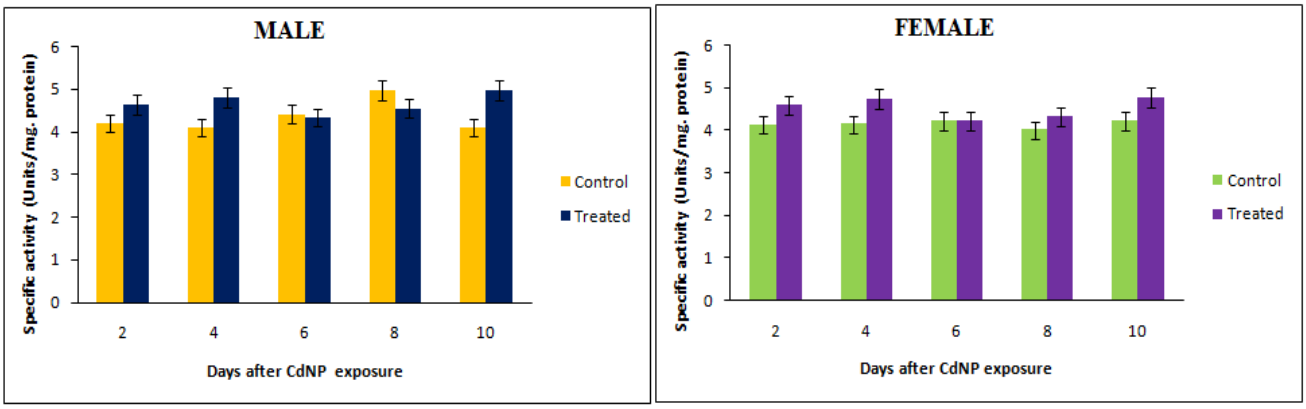

Figure 8. Catalase activity (CAT) in muscle of male and female crab, *S. olivacea* after exposure of CdNP

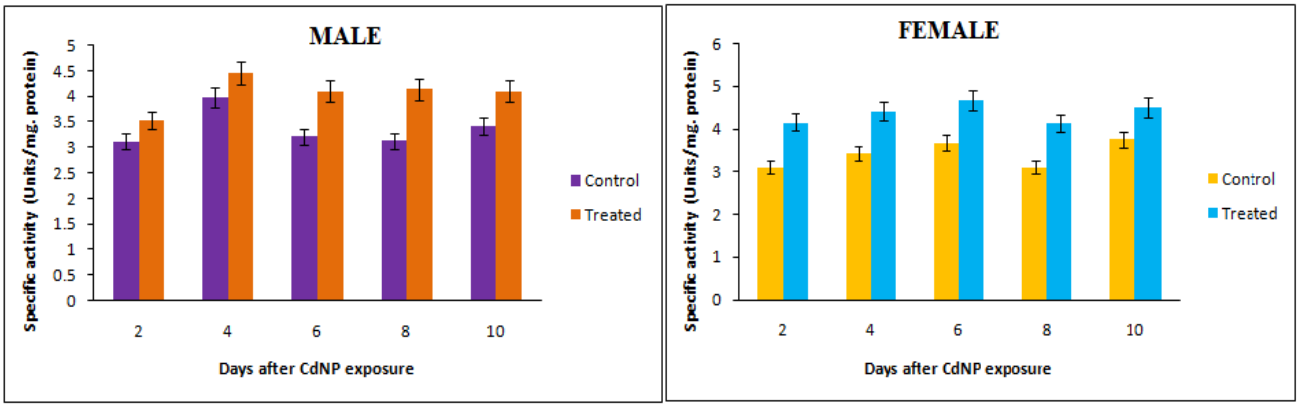

Figure 9. Superoxide dismutase (SOD) activity in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

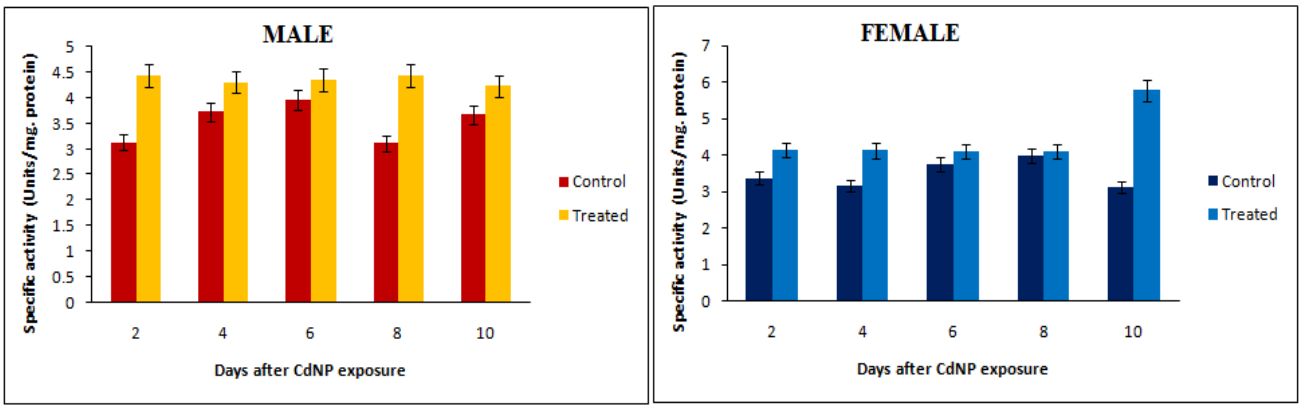

Figure 10. Glutathione Peroxidase (GPx) activity in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

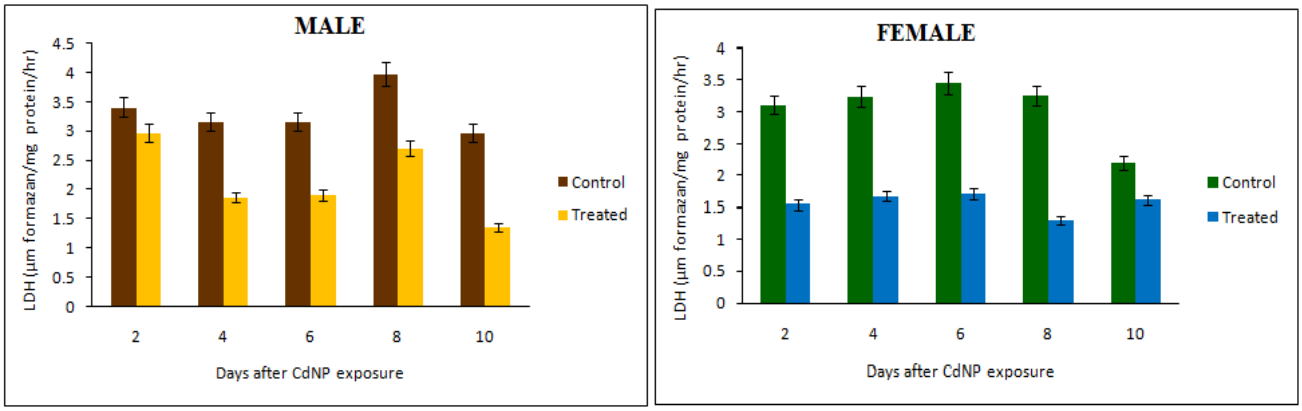

Figure 11. Lactate dehydrogenase (LDH) activity in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

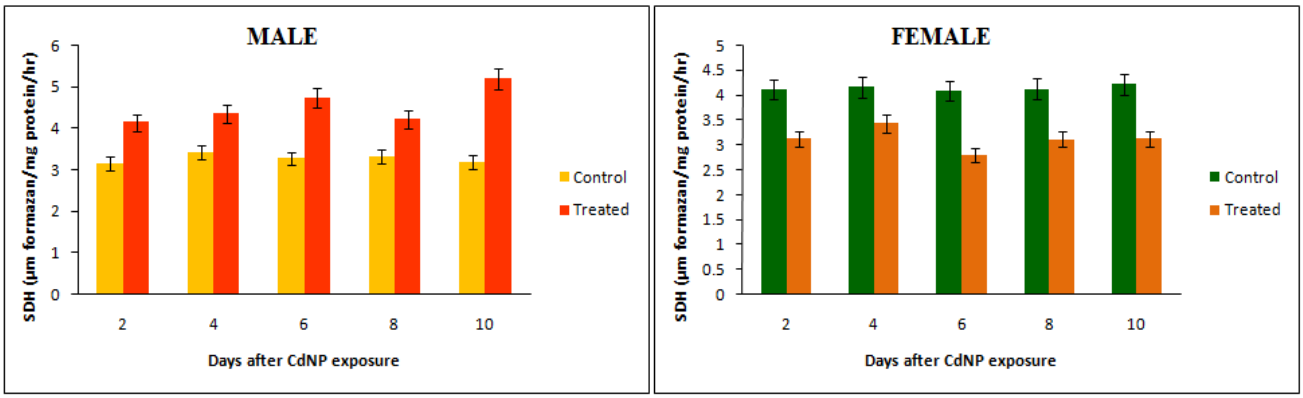

Figure 12. Succinate dehydrogenase (SDH) activity in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

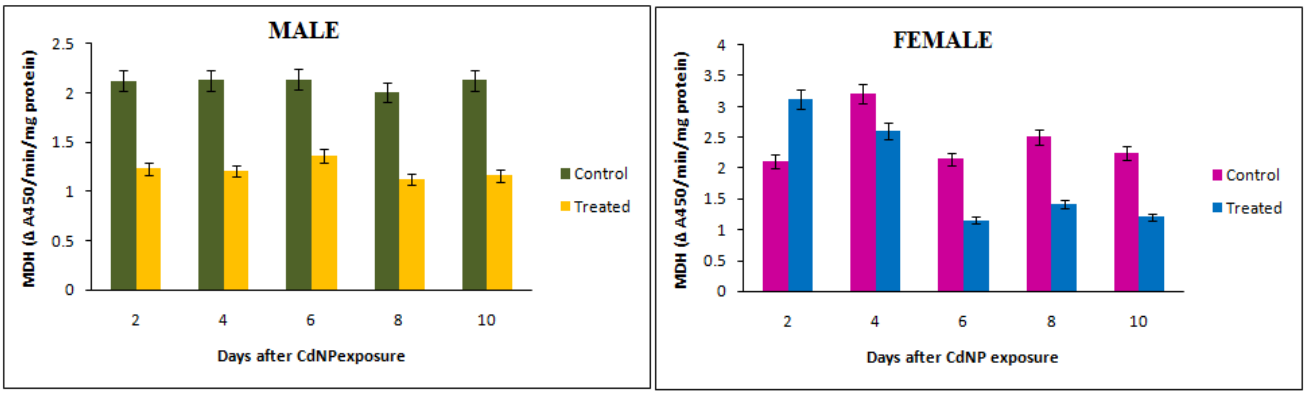

Figure 13. Malate dehydrogenase (MDH) activity in muscle of male and female crabs, *S. olivacea* after exposure of CdNP

A maximum of upto two and four fold increase in GPx activity was recorded in male and female crabs respectively compared to their respective controls.

Effects of CdNP on Lactate Dehydrogenase (LDH) activity

Results of LDH activity of *S. olivacea* exposed to Cadmium nanoparticle (20ppm) were presented in Fig.56 & 57. In general, CdNP exposure resulted in increased LDH activity in muscles than in control crabs. In males, SDH activity started increasing in exposed crabs right on day 2 and reached maximum on day 10 of exposure (Figure 11). Similarly, in the case of female LDH activity started decreasing in exposed crabs right on day 2 and reached lowest on day 10 of exposure. A maximum of upto five fold and three fold decrease in LDH activity was recorded in male and female crabs respectively compared to control. With reference to LDH activity, both male and female crab showed susceptibility to CdNP.

Effects of CdNP on Succinate Dehydrogenase (SDH) activity

Results of SDH activity of *S. olivacea* exposed to Cadmium nanoparticle (20ppm) were presented in 12. In general, CdNP results in decreased SDH activity in the muscles than in control crabs. In males, SDH activity started decreasing in exposed crabs right on day 2 and reached lowest on day 10 of exposure. A maximum of upto two fold decreases in SDH activity was recorded in male crab compared to control on day 10 after exposure. Similarly, in the case of female SDH activity started increasing on day 2 and decreased on day 10 of exposure.

A maximum of upto two and three fold decrease in SDH activity was recorded in male and female crabs respectively compared to control. With reference to SDH activity, female crab showed more susceptibility to CdNP compared to male crabs.

Effects of CdNP on Malate Dehydrogenase (MDH) activity

Results of MDH activity in the muscles of *S. olivacea* exposed to Cadmium nanoparticle (20ppm) were presented in figure 13. In general, CdNP resulted in decreased MDH activity than in control crabs. In males, MDH activity started decreasing right on day 2 compared to control and reached lowest compared to control on day 10 of exposure. Similarly, in the case of female also MDH activity started decreasing on day 2 compared to control and reached lowest compared to control on day 10 of exposure.

DISCUSSION

The present study was focused to study the effect of Cadmium nanoparticle (CdNP) on the histology and biochemical changes in mud crab *Scylla olivacea*. Casalino *et al.,* (1997) showed that Cd mainly accumulated in the cytosol (70%), followed by the nucleus (15%) with the lowest accumulation found in the mitochondria and the endoplasmic reticulum. In the present study, both nuclei and mitochondria were sensitive to acute CdNP toxicity. Mitochondria are the primary source of ROS and the target of excessive ROS generation. The present study showed decreased activity of mitochondrial enzymes which re ect the disturbances of oxidation–reduction processes taking place in this organelle. Oxidative stress has been

considered as one of the basic events involved in cell and tissue damage. Radicals can cause damage to cardinal cellular components such as lipids, proteins and nucleic acids leading to subsequent cell death by modes of necrosis or apoptosis (Gilgun-Sherki *et al.,* 2002). ROS-mediated lipid peroxidation is one of the major mechanisms of damage. It has been shown that antioxidant defense systems protect cells from Cd-induced toxicity (Ognjanovic *et al.,* 2006). Antioxidant enzymes like SOD, CAT and GPx constitute the major defensive system against ROS and the decrease in their activities contribute to the oxidative insult on the tissue. Signi cant changes in the activity of antioxidant enzymes during the treatment of crabs with CdNP were observed.

SOD and CAT are the two primary enzymes for radical scavenging, which are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis and their activities are related to the status of the organisms affected by different factors including dietary nutrition, environmental factors etc. Therefore, significantly higher SOD and CAT activities might indicate that the stress resulted in an accumulation of radicals to a higher level in crustaceans (Winston & Giulio, 1991). Therefore, the enhanced activities of both SOD and CAT may enable crabs to maintain health by scavenging the radicals produced. The adaptive mechanism may be partially explained by the increasing activities of SOD and CAT for scavenging the radicals produced in a certain extent (Messaoudi *et al*., 2010). The alterations in SOD activity may depend on various factors such as dose, exposure time, type of metals/ toxic compounds and the physiological state of the animal (Yalin *et al.,* 2006). CAT and GPx catalyze the conversion of hydrogen peroxide to water, thus CAT and GPx could reduce the tissue injury by removing the hydroxyl radicals. Previous studies reported that singlet oxygen, superoxide and peroxyl radicals were inhibitors of CAT (Kono and Fridovich, 1982; Escobar *et al.,* 1996). This is probably a consequence of the intracellular accumulation of ROS with subsequent development of heart injury. However, the higher GPx activity might be a result of de novo synthesis, possibly induced by increased oxidative stress in the heart (Gehringer *et al.,* 2004).

An increase in GPx reduced the harm to organism by H^+, offsetting the possible adverse effects that would otherwise arise from the lack of increased CAT activity at this time. After 8 day of Cd exposure, a signi cant reduction in GPx and CAT activity was observed. The decreased activity of GPx can be explained by competition of Cd for the thiol groups in metallothioneins and GPx (Waisberg *et al.,* 2003). Alternatively, loss of GPx activity could be correlated to depletion of selenium by Cd (Zumkley, 1988) or due to decline in GSH concentration (Rao *et al.,* 2005). The decreased activities in GPx and CAT, along with the increased activity in SOD, suggest that there must be an oxidative stress in response to acute intoxication of Cd and that an oxidative damage was induced, consistent with the histopathological damage at the CdNP concentrations (20 ppm/kg body weight). The LDH, SDH and MDH activities are marker for tissue damage in aquatic animals (Ramesh *et al.,* 1993), muscle harm (Balint *et al.,* 1997) and hypoxic condition (Das et al., 2004) and good diagnostic tool in toxicology. LDH is an enzyme involved in conversion of pyruvate to lactate in glycolysis thereby leading to the formation of lactic acid. Thus formed lactate is an important gluconeogenic substrate which can be used to cope up with the high and rapid demand of energy due to stress. *U.annulipes* treated with sublethal concentration of cadmium and mercury showed elevation of LDH activity (Suresh, 2001) and *S. hydrodroma* in treatment with copper and zinc (Jayakumar, 2002). According to many workers *O.senex* showed increased LDH and decreased SDH activity in response to sumithion (Reddy *et al.,* 1983). In accordance with the previous findings the present study also demonstrated that increased LDH and decreased SDH and MDH may contribute the energy requirement of the fiddler crab, *S.Olivacae*. Imbalance in the metabolic enzymes may decline the level energy in the organ system and in response to cadmium exposure the increase in LDH activity and declined SDH, MDH activity may help to maintain a balance in the energy requirement of the organism during the exposure to sublethal concentration of cadmium nano particles.

Conclusion

Imbalance in the metabolic enzymes may decline the level energy in the organ system and decreased metabolic activity may also help to maintain a balance in the energy requirement of the organism during the exposure to sublethal concentration of cadmium nanoparticles. So the overall results concluded that the heavy metals in nanoscale may affect the eco-balance in the aquatic system which is reflected in the decrease in the population of the aquatic animals exposed the heavy metals intoxication.

Acknowledgments

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