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CARBON NANOTUBE INDUCED CHANGES IN SOME METABOLIC ENZYMES OF THE FISH, *CHANNA PUNCTATUS*

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ABSTRACT : The use of nanoparticles is growing exponentially, but they are hazardous to biological world. Nanoparticles released in aquatic environment may affect cellular and biochemical functions and in turn, reduce fish productivity. Carbon nanotube is a nanomaterial which is mainly used for water purification, but they may cause toxicity in aquatic species. Hence the effects of carbon nanotubes on some metabolic enzymes of the freshwater fish *Channa punctatus* were investigated. Treatment of carbon nanotubes declined the activity of succinate dehydrogenase (SDH), cytoplasmic malate dehydrogenase (cMDH) and lactate dehydrogenase (LDH) in liver, brain, gill and kidney of the fish. Maximum decrease in SDH activity was in gill followed by kidney, brain and liver. The decline in cMDH activity was maximum in kidney and minimum in brain. Likewise, reduction in LDH activity was maximum in brain and minimum in gill. The tissue-specific reductions in activity of enzymes may be assigned to the differences in nature of different organs. Among these enzymes maximum decline was in activity of cMDH reflecting inhibition in gluconeogenic and lipogenic capacity of fish. The effects of carbon nanotubes on activity of these metabolic enzymes were concentration-dependent. The present findings indicated that the carbon nanotubes are toxic to the cellular and subcellular enzymes and their uses should be restricted to save the health of fish.

Key words : Carbon nanotube, SDH, cMDH, LDH, fish.

INTRODUCTION

Incorporation of undesirable and harmful substances in nature is pollution. Water pollution, due to different types of waste disposal has become a very serious global problem. Recently, the use of engineering nanoparticles has increased drastically due to their dynamic and wide applications for various purposes. In spite of beneficial role of nanoparticles, they have been reported to harm biological world. For instance various nanoparticles like carbon, silver, gold, zinc etc. are utilized in different fields but they may be harmful to biological system (Helfenstein *et al*, 2008). In the last two decades researchers have shown more interest in nanoscience because of unique physical and chemical properties of nanoparticles. It has been reported that nanoparticles are toxic to bacteria, algae, invertebrates, fish and mammals (Handy *et al,* 2008). Fishes are one of the important sources of healthy diet but they are severely affected by water pollution (Hoo *et al*, 2004; Kumar and Achyuthan, 2007; Srivastava and Srivastava, 2008). Nanoparticles cause oxidative stress, cytotoxicity and inflammatory responses in fish and other aquatic organisms (Abirami *et al*, 2017). The harmful effects of nanoparticles in aquatic environment are related to their ability to cause oxidative damages through generation of free radicals. The generation of reactive oxygen species (ROS) depends on size, shape and nature of nanoparticles. Due to extremely small size nanoparticles easily penetrate the cell membrane and affect cellular metabolism. Many nanoparticles interact with the cell membrane, disrupt the membrane functions like ion transportation mechanisms and process of signal transduction. The chemical nature and physical properties of the nanoparticles may lead to cytotoxic changes (Hondroulis *et al*, 2014).

The release of carbon nanotubes and other nanoparticles in environment is increasing rapidly and they are affecting biological system and damaging the aquatic organisms including crustaceans and fishes (Kishore *et al*, 2014). Carbon nanotubes (CNTs) are the third most useful nanoparticles after silver and zinc among consumer products (Sohn *et al*, 2015). They provide economic and social benefits to human society, but their adverse effects on the organisms and environment have gradually emerged. Carbon nanotubes not only cause the embryonic death of animals, but also inhibit the growth of animals. CNTs released from composite materials are also considered as potential source of environmental pollution (Chen *et al*, 2017b; Laux *et al*, 2018). Multi walled carbon nanotubes (MWCNTs) reduce microbial enzyme activity, biomass, change the bacterial composition and cause

microbial cell death when at high concentration (Chan *et al*, 2019). They produce respiratory toxicity including inflammatory changes, lung fibrosis and promotion of lung adenocarcinoma (Muller *et al*, 2005; Ryman-Rasmussen *et al*, 2009a; Sargent *et al*, 2014; Smith *et al*, 2019). Exposure of single walled carbon nanotubes (SWCNTs) significantly reduces the activity of acetylcholine esterase in brain of zebrafish (Rocha *et al*, 2019).

The respiratory and ion transport surface is greater than 60% of the total surface area in fish so they provide large area for interaction of nanoparticles (Rombough and Moroz, 1997; Karthigarani and Navaraj, 2012). The MWCNTs may cause oxidative stress and apoptosis in *Rainbow trout* (*Oncorhynchus mykiss*) in both *in vitro* and *in vivo* conditions (Lee *et al*, 2015). Genotoxicity induced by CNTs may be the results of direct interaction of the nanoparticles with the genetic materials, indirect damage by reactive oxygen species, the release of toxic ions, interaction with enzymes and other constituents (Wang *et al*, 2015, 2016; Girardi *et al*, 2016; Cimbaluka *et al,* 2018). Enzymes are biological catalysts which control all metabolic processes hence, variation in enzyme activity may effect activity of organisms (Govindu, 2016). Succinate dehydrogenase (SDH) is one of the key enzymes of Kerbs cycle which catalyzes reversible conversion of succinate to fumarate in mitochondria. It is the only enzyme that participates in both the Krebs cycle and electron transport chain (Govindu, 2016). Cytoplasmic malate dehydrogenase (cMDH) catalyzes interconversion of oxaloacetate to malate in cytoplasm. However, lactate dehydrogenase (LDH) is a cytoplasmic enzyme which catalyzes interconversion of pyruvate to lactate and is responsible for energy production under anaerobic condition.

Continuous exposure of carbon nanotubes (CNTs) to fish may lead to health problem and mortality. CNTs may cause toxicity rendering biochemical and enzymatic changes in fish. Toxic effects of carbon nanotubes have been studied in some species of fishes including *Oncorhynchus myskiss*, *Poecilia reticulate*, *Nile tilapia* (Smith *et al*, 2007; Garcia *et al*, 2014; Lukhele *et al*, 2015 and Nymbe *et al*, 2015). Certainly the ill-effects of carbon nanotubes need evaluation at enzymatic level. In this view, the present study was designed to elucidate the toxic effects of carbon nanotubes on succinate dehydrogenase, cytoplasmic malate dehydrogenase and lactate dehydrogenase of liver, brain, gill and kidney of an economically important freshwater food fish, *Channa punctatus*.

MATERIALS AND METHODS

Fish

Healthy and adult specimens of an air breathing freshwater fish, *Channa punctatus* (150-160 g weight; 15-20 cm body length) were collected from the local fish markets of Jodhpur, India. The collections were done from November to February. They were acclimatized to laboratory conditions at least for one week prior to experimentation.

Chemicals

Chemicals were either of Sisco Research Laboratory (India) or Sigma Chemical Company (USA). Carbon nanotubes (CNTs) were purchased from Reinste Nano Ventures Pvt. Ltd, Noida, India. They were having 1-10μm length, 5-20 nm outer diameters, 2-6nm inner diameter and 95% purity.

CNT exposure

Fishes were divided into two groups. Out of these two groups one was control which did not receive carbon nanotubes and others were treated groups which received separately three different sublethal concentrations (100ppm, 200ppm, 300ppm) of multi walled carbon nanotubes (MWCNT). They were exposed to MWCNT for 14 days. Water of fish container was changed on every four days and fresh concentration of MWCNT was maintained in the container. Fish feeding was stopped during CNT treatment.

Enzyme extraction

The fish was sacrificed by decapitation after anaesthesization on ice. The liver, brain, gill and kidney were removed and washed immediately with 0.6% of saline and cleaned properly. A 10% homogenate (w/v) was prepared in ice-cold 0.1 M sodium phosphate buffer (pH 7.5) using Potter-Elvehjem homogenizer. The homogenates were centrifuged at 700g for 10min in a high speed refrigerated centrifuge to remove cell debris. The supernatant was decanted and centrifuge at 12,000g for 15 min to get the mitochondrial pellet. The resulting supernatant was taken as cytoplasmic fraction for the assay of cytoplasmic malate dehydrogenase (cMDH) and lactate dehydrogenase (LDH). The mitochondrial pellet was washed in 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged at 12,100g for 10 min. Finally, the mitochondrial pellet was suspended in the above buffer and homogenized at high speed. The homogenized suspension was recentrifuged at 21,000g for 15 min to remove particulate matter. The resulting supernatant was taken as mitochondrial fraction for the assay of succinate dehydrogenase (SDH).

Enzyme assay

SDH (EC 1.3.99.1)

Activity of SDH was estimated with the help of the method described by Nachlas *et al* (1960). The reaction mixture contained sodium phosphate buffer (0.1 M; pH 7.5), sodium succinate (40 mM; pH 7.4) and INT (2,4 Iodo phenyl-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride; 4 mM) and enzyme. The volume of reaction mixture was 3 ml. The reading was taken at 495 nm in UV-Visible spectrophotometer.

MDH (EC 1.1.1.37)

The procedure adopted for the assay of cytoplasmic malate dehydrogenase (cMDH) was of Schwantes and Schwantes (1982). MDH activity was measured in a medium containing 0.1 M sodium phosphate buffer (pH 7.5), 0.4 mM oxaloacetate and 0.12 mM NADH. Total volume of reaction mixture was 3 ml. The absorbance was taken with the help of UV-Visible spectrophotometer. Decrease in absorbance was recorded at 340 nm.

LDH (EC 1.1.1.27)

The LDH was assayed according to the method of Foster and Moon (1986). LDH activity was measured in a medium containing 0.1M sodium phosphate buffer (pH 7.5) pyruate (4 mM) and NADH (0.2 mM). Total volume of reaction mixture was 3 ml. The absorbance was taken with the help of UV-Visible spectrophotometer. Decrease in absorbance was recorded at 340 nm.

Statistical analysis

One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) were performed using SPSS statistical package. The level of significance was set at 0.05.

RESULTS

The one way ANOVA showed significant variation in activity of succinate dehydrogenase (SDH), cytoplasmic malate dehydrogenase (cMDH) and lactate dehydrogenase (LDH) of liver, brain, gill and kidney of the freshwater food fish (*Channa punctatus*) with respect to changes in concentrations of carbon nanotubes (CNTs) (Figs. 1-3). The SDH activity decreased significantly (<0.05) in gill followed by kidney, brain and liver in response to 300 ppm CNT treatment. Similarly, the activity of cytoplasmic MDH also decreased significantly (<0.05) in kidney followed by gill, liver and brain after exposure of fish to CNT. Reduction in activity of LDH was maximum in brain and minimum in gill.

The exposure of fish to different concentrations of carbon nanotubes (100, 200 and 300ppm) for 14 days significantly declined the SDH activity of liver (13%, 29%, 52%), brain (29%, 42%, 56%), gill (37%, 51%, 66%) and kidney (28%, 42%, 58%), respectively. However, the values of SDH activity of brain, gill and kidney did not differ significantly between 100 and 200 ppm as well as between 200 and 300 ppm of carbon nanotube treatment. Similarly, decreases in cMDH of liver (14%, 37%, 62%), brain (32%, 43%, 58%), gill (27%, 39%, 63%) and kidney (21%, 32%, 67%) were observed in response to treatment of different concentrations (100, 200, 300ppm) of carbon nanotubes, respectively. Like SDH, the activity of cMDH of brain, gill and kidney did not differ significantly at 100 and 200 ppm as well as between 200 and 300 ppm nanotube treatment. A gradual reduction in LDH activity of liver (17%, 21%, 34%), brain (7%, 18%, 40%), gill (4%, 13%, 34%) and kidney (19%, 26%, 36%) were noticed after treatment of 100, 200 and 300 ppm carbon nanotubes. Similar to SDH and cMDH, the activity of LDH of liver, brain, gill and kidney did not different significantly at 100 and 200 ppm as well as between 200 and 300 ppm of nanotubes.

Maximum decrease (66%) in SDH activity was obtained in gill in response to 300 ppm carbon nanotube treatment. It was close to SDH reduction in kidney (58%). While minimum decrease was observed in liver (13%) after treatment of 100 ppm nanotube. Maximum decrease (67%) in cMDH was obtained in kidney in response to 300 ppm carbon nanotube. It was almost similar to the cMDH reduction in gill (63%). Whereas minimum (14%) decrease was observed in liver after treatment of 100 ppm nanotube. The effect of carbon nanotube on LDH activity showed maximum decline (40%) in liver at 300 ppm and minimum but insignificant decline (4%) in gill at 100 ppm nanotube concentration.

DISCUSSION

The present study demonstrated the toxic effects of carbon nanotubes (CNTs) on succinate dehydrogenase (SDH), cytoplasmic malate dehydrogenase (cMDH) and lactate dehydrogenase (LDH) in liver, brain, gill and kidney of the freshwater fish, *Channa punctatus* (Figs. 1-3). These metabolic enzymes showed significant reductions in their activity in the osmoregulatory (gill and kidney) and non-osmoregulatory (liver and brain) tissues of the fish. It indicated the suppression of metabolic process in the fish exposed to CNTs. The negatively charged surfaces of carbon nanotubes might be the main cause for the decrement of enzymatic activities (Zhang *et al*, 2011). Cellular structures and functions are influenced by physiochemical characteristics of the carbon nanotubes. Carbon nanotube may affect the expression of protein, which are involves in cell to cell interaction (Eldawud *et al*, 2018). The main routes of uptake of

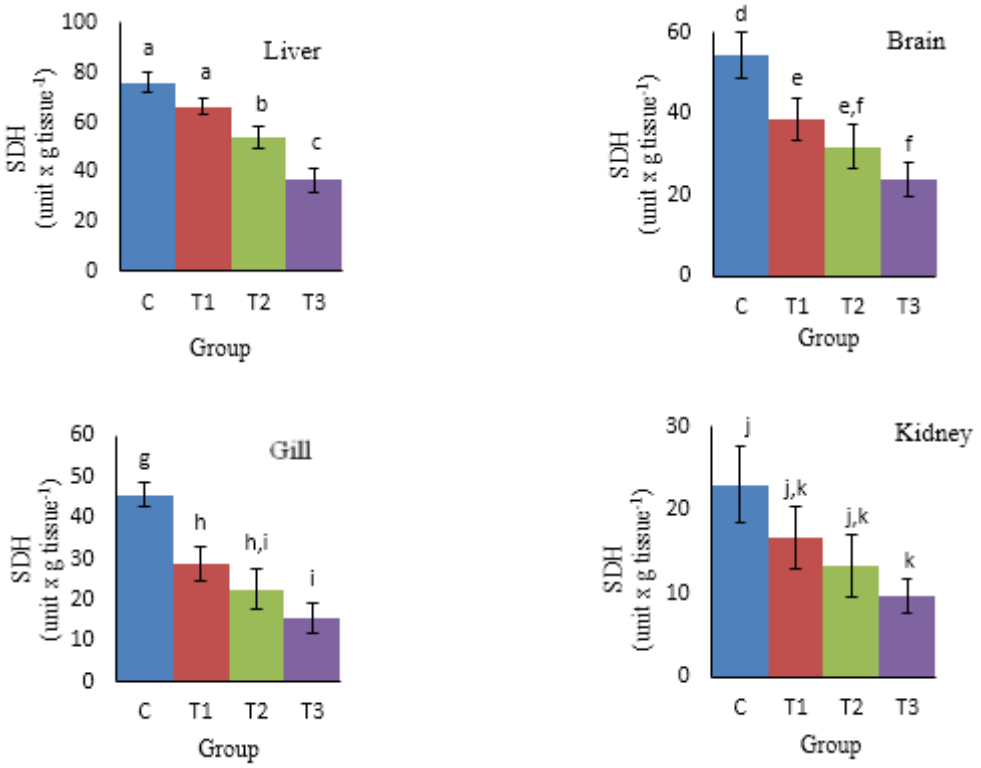

Fig. 1 : Effect of carbon nanotube (CNT) on activity (unit x g tissue^{-1}) of succinate dehydrogenase (SDH) in liver, brain, gill and kidney of the freshwater fish, *Channa punctatus*. C, Control group did not receive CNT; T_1, 100 ppm CNT treated group; T_2, 200 ppm CNT treated group; T_3, 300 ppm CNT treated group. The values with different letters are significantly different.

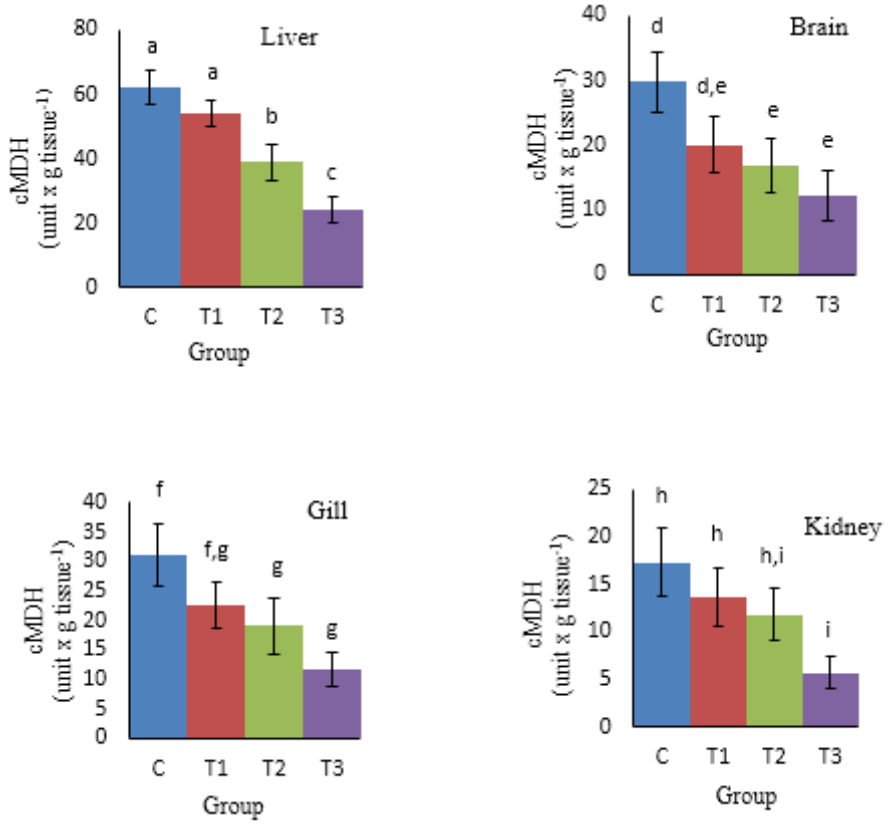

Fig. 2 : Effect of carbon nanotube (CNT) on activity (unit x g tissue^{-1}) of cytoplasmic malate dehydrogenase (cMDH) in liver, brain, gill and kidney of the freshwater fish, *Channa punctatus*. C, Control group did not receive CNT; T_1, 100 ppm CNT treated group; T_2, 200 ppm CNT treated group; T_3, 300 ppm CNT treated group. The values with different letters are significantly different.

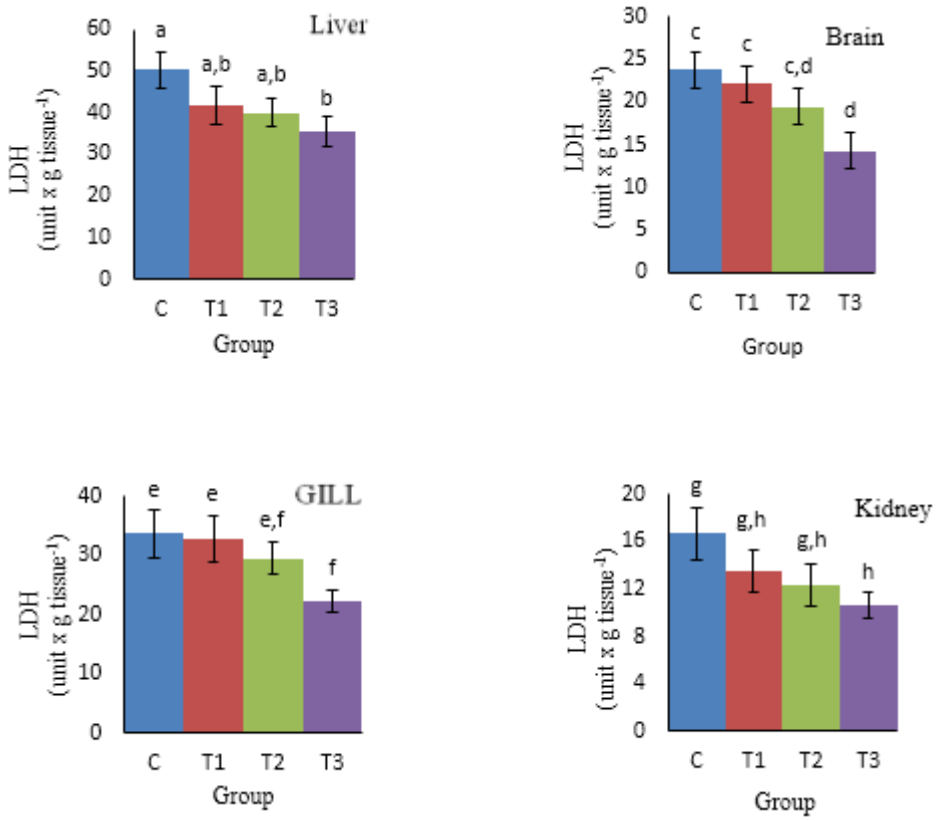

Fig. 3 : Effect of carbon nanotube (CNT) on activity (unit x g tissue^{-1}) of lactate dehydrogenase (LDH) in liver, brain, gill and kidney of the freshwater fish, *Channa punctatus*. C, Control group did not receive CNT; T_1, 100 ppm CNT treated group; T_2, 200 ppm CNT treated group; T_3, 300 ppm CNT treated group. The values with different letters are significantly different.

nanoparticles by fish are skin and gut. Nanoparticles may enter into fish body by diffusion process through cell membrane (Lin and Xing, 2007), endocytosis (Kim *et al*, 2006) and adhesion (Geiser *et al*, 2005). Further the freshwater fish engulf water proportional to their body mass through which nanoparticles enter inside the gut (Lukhele *et al*, 2015). Enzyme activity may be increased or decreased due to organ destruction and molecular denaturation by toxicants (Valarmathi and Azariah, 2003).

Toxicants alter the mitochondrial structure and decrease the activity of TCA cycle enzymes in organs (Racicool *et al*, 1975, Bag *et al*, 1999). Suneetha (2012) described decrease in SDH activity in brain, gill, kidney and liver of the freshwater fish, *Labeo rohita* after exposure to lethal and sublethal concentration of endosulfan and fenvalerate for 15 days. Here aerobic pathway might have turned towards anaerobic to meet the increased energy demand during toxic stress. Sangeetha *et al* (2015) showed that after exposure of cadmium nanoparticles significantly decrease the activity of SDH, MDH and LDH in gill of mud crab *Scylla Olivacea*. It was due to progressive injuries of gill and alterations in biochemical defence mechanism after nanoparticle exposure. Wu and Zhou (2013) reported that silver nanoparticles declined the LDH activity in liver of *Oryzias latipes*. These finding supports the present observations of CNT-induced decline in SDH and LDH activity of liver, brain, gill and kidney of *C. punctatus*. Griffitt *et al* (2009) suggested that metallic nanoparticles produce toxicity in aquatic organisms that is largely due to particulates as opposed to release of dissolved ions. Linhua *et al* (2009) suggested that oxidative stress is common pathway of toxicity. Vander *et al* (2003) reported that fish exposed to titanium oxide (TiO_2) nanoparticles deplete super oxide dismutase (SOD) activity. It may be used as an indicator of free radical formation in cell and also reflects that the antioxidant defense system is affected by reactive oxygen species (ROS). Production of reactive oxygen species affects the growth and survival of aquatic fauna (Filho, 1996; Pandey *et al*, 2003).

Afifi *et al* (2016) found that exposure of Nile tilapia (*Oreochromis niloticus*) and Redbelly tilapia (*Tilapia zillii*) to silver nanoparticles decreased the activity of super oxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) in brain. Nanoparticle toxicity might be stimulating the generation of ROS through disruption of intracellular metabolism (Warheit *et al*, 2006;

Long *et al*, 2006) or damage in antioxidant defence system (Brown *et al*, 2004) causing changes in protein, lipid, DNA and carbohydrate concentration (Kelly *et al*, 1998). Antioxidant defence mechanisms have two pathways viz., enzymatic and non-enzymatic defensive mechanisms. The non-enzymatic antioxidant mechanism consists of vitamin C, vitamin E, carotenoids, amino acids and the enzymatic antioxidant mechanism includes SOD, CAT, GR and peroxidase etc. (Martinez-Alvarez *et al*, 2005). Like the present observations to some extent, Reddy *et al* (2013) described that exposure of Indian major carp, *Catla catla* to silver nanoparticles significantly decreases the activity of SDH with a significant increase in LDH activity of gill, liver and kidney in bacteria intoxicated fish as compared to control. But a significant increase in the activity of SDH with a significant decrease in the activity of LDH in gill, liver and kidney were observed in bacteria intoxicated fish exposed to silver nanoparticles as compared to bacteria intoxicated fish. Nanoparticle-induced expression of cytochrome P450 monooxygenase system suggests possible increase in oxidative metabolism in gill (Scown *et al*, 2010).

Carbon nanotube-induced decrease in SDH, cMDH and LDH activity indicated reduction in aerobic capacity, gluconeogenesis and lipogenesis, and increase in anaerobiosis respectively. Decline in activity of these enzymes might disrupt intracellular metabolism (Suneetha, 2012; Reddy *et al*, 2013; Wu and Zhou, 2013 and Sangeetha *et al*, 2015) and stimulate formation of ROS leading to oxidative stress in the freshwater fish, *C. punctatus.* Thus continuous use of carbon nanotube may severely effect enzymatic activity and hamper fish production in aquatic ecosystem. Consumption of carbon nanotubes by fish may affect human health too via ecological cycling and biological magnification. Therefore, the use of carbon nanotubes should be restricted to protect aquatic fauna and human health.

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