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Histopathological changes in the eyes, gills and muscles of Indian major carp *Catla catla* (*Hamilton, 1822*) exposed to Cadmium nanoparticles

[1] S Sangeetha, [*2] S Deeparani
[1] Assistant Professor, PG & Research Department of Zoology, Pachaiyappas College, Chetpet, Chennai, Tamil Nadu, India
[2] Assistant Professor, PG & Research Department of Zoology, Ethiraj College for Women, Chennai, Tamil Nadu, India

Abstract
Heavy metals are essential for aquatic animals in lower concentration. However, at high concentration levels, they accumulate in different organs, damage tissues and interfere with the normal growth and proliferation. More than one hundred million people are at high risk of elevated cadmium exposure, mainly via drinking water, as well as by the air born metalloid in the areas with coal burning and industrial emissions. Consumption of the cadmium through contaminated fishes collected from the polluted waters might also contribute to bioaccumulation of cadmium in human beings. In the present study the toxicity of Cadmium nanoparticle exposure was experimented in Indian major carp, *Catla catla*. The LC50 value was assessed as 20ppm/kg of body weight. The histological changes in the eyes, gills and muscles were studied. The eye tissues showed disorganization of corneal cuticle and formation of corneal cones and constriction of internal and external medulla and complete disorganization of basement. The changes observed in the gill of *Catla catla* were swelling, fusion of lamellae, severe erosions of epithelial layer and high mucus secretion. While muscle tissues showed marked thickening and separation of muscle bundles, haemolysis, necrosis, lesions with reduced compactness and pronounced intramuscular oedema with minor dystrophic changes.

Keywords: gills, muscles, cadmium, nanoparticles, toxicity

1. Introduction
Heavy metals are widely used in various industries and considered as common water pollutants. When the amount of heavy metals in a medium, reach to more than a certain limit, it becomes toxic for those animals that live in the environment (Alkarkhi *et al.*, 2009). Low concentrations of some heavy metals are essential for aquatic animals. However, at high concentration levels, they accumulate in different organs, damage tissues and interfere with the normal growth and proliferation (Alkarkhi *et al.*, 2009). That is why knowledge regarding the toxicity of heavy metals to aquatic organisms is of paramount importance (Rathore and Khangarot, 2003). All aquatic organisms are directly or indirectly affected by the physical characteristics of their environment, especially the chemical composition of the water (Gillis *et al.*, 2008).
A number of investigators have reported that the toxic effect of heavy metals on freshwater organisms is affected by water hardness (Kim *et al.*, 2001; Markich *et al.*, 2006). Studies have also shown that environmental factors play an important role in modifying the toxicity of metals (Vedamanikam and Shazilli, 2008). These reports show that increasing water hardness reduces the toxic effect of metals in aquatic organisms. The effects of metals on aquatic organisms have been the subject of numerous investigations (Martins *et al.*, 2004; Kim *et al.*, 2001; Vedamanikam and Shazilli, 2008). Although many research have been conducted to assess the toxicity of heavy metals in algae, however, the number of studies dealing with the toxic effect of heavy metal on aquatic animals including fish are limited (Harmon *et al.*, 2005).
Cadmium contamination in drinking water has become a significant concern in Bangladesh, West Bengal, India, China, Mongolia, Nepal, Cambodia, Myanmar, Afghanistan, Korea, and Pakistan (Mukherjee *et al.*, 2004). It has also been reported to contaminate marine environments (Elia *et al.*, 2000), freshwater environments (Ciardullo *et al.*, 2010) and groundwater (Alam *et al.*, 2002). Contamination of the aquatic environment by cadmium has increased during recent years primarily due to anthropogenic sources (Horacio *et al.*, 2006; Bears *et al.*, 2006).
More than one hundred million people are at high risk of elevated cadmium exposure, mainly via drinking water, as well as by the air born metalloid in the areas with coal burning and industrial emissions. Consumption of the cadmium through contaminated fishes collected from the polluted waters might also contribute to bioaccumulation of cadmium in human beings. Hence it is of immense importance to know the cadmium induced damages in the different organ systems of fishes used for human consumption.

2. Materials and methods
2.1 Collection of experimental animal
Catla catla fishes were collected from Poondi Lake, Thiruvallur dist, Tamil Nadu, India. Fishes were maintained under controlled laboratory conditions. All the fishes were acclimated for at least two weeks and fed with libitum fish diet in a laboratory conditions at the ambient, uncontrolled temperature of 28±2°C under the (12h:12h) light: dark conditions. During the acclimatization the fishes were fed with feed daily in the evening, uneaten feed was removed next day morning followed by 100% water exchange.

2.2 Acute toxicity test

The acute semi static toxicity test was carried out according to the standard methodology of the *Food and Agriculture Organization* (FAO) (Ward and Parrish, 1982; Reish and Oshida, 1987) [32, 30] and the American Public Health Association (APHA, 1992) [2]. Fishes were acclimatized for 14 days. Semi static toxicological bioassays were carried out for 96 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 mixed intraperitonially per kg of fish weight. Three replicates of at least 10 animals were exposed to the above-stated concentrations. The criteria to determine death was the complete absence of movement once the animals were gently touched with a glass rod. Mortality was recorded every 24 h, a period of time after which dead fishes were removed. The experimental conditions (temperature, salinity, and pH) of the toxicity test were similar to those found in the environment during the period. To match the environmental conditions, an average of these parameters was used. A probit analysis was used to estimate the concentration and 95% confidence limits of CdNP that kills 50% of the exposed fish (LC_{50}).

2.3 Cadmium nanoparticle treatment

After, standardization of LC_{50} value, a single concentration of 20ppm/kg of body weight was used for further experiments. Major Indian carp *Catla catla* was acclimatized in tanks and the temperature was maintained at 27°C. Water was changed daily and aquaria were cleaned thoroughly, and Fishes were fed commercial fish feed. After acclimatization, healthy adult male and female fishes with a homogeneous size (width 14-16cm, weight 200-300g) were selected for control and Cadmium nanoparticle (20ppm/kg / weight) treatment. The acute exposure lasted for 5 days. During the experiment, fishes were fed and dead animals were removed in time.

2.4 Histopathological study

2.4.1 Light Microscopic Analysis

Fishes were exposed to Cadmium nanoparticles at 20ppm for 5 days. Sampling was done on the every 24 hr. of exposure; two fishes in each group were sacrificed. The gills, eyes and muscles of representative fishes from each test and control group were dissected out and fixed in Davidson's fixative for 24hrs. The preserved tissues were processed by a routine histological method, dehydrated in alcohol series and embedded in paraffin wax. They were cut into sections of 6 mm thickness by a rotary microtome (Weswox, MT1090:1090A, India). The thin sections of the tissues were stained by haematoxylin and eosin for observation by the Nikon Bright field Transmission microscope with Koechler illumination and automatic exposure unit was used (Bernet *et al.*, 1999) [5].

2.4.2 Scanning Electron Microscopic Analysis

Three to six small pieces of 1 mm^2 in size of the following tissues of both male and female were taken: Gills, eyes and muscles were fixed in 2.5% glutaraldehyde in 0.1 % phosphate buffered saline (pH 7.4) for about 12 h and the fixed samples were immersed in a series of ethanol (10%, 25%, 50%, 75% and 100%) for dehydration and coated with gold. All samples were examined with Scanning Electron Microscope (SEM) (Hitachi S3400, Japan).

3. Results and discussion

3.1 Toxicity Assessment of CdNP on *Catla catla*

Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Farombi & Adedlowo, 2007) [3, 11]. One of the most important characteristics of toxic pollutants such as metals is that they can be accumulated in organs of the organisms (Palniappan and Karthikeyan, 2009) [27]. Fish have been largely used in the evaluation of the quality of aquatic systems. These organisms are often at the top of the aquatic food chain and may concentrate large amount of metals from the surrounding waters (Rajkowska and Protasowicki, 2011) [28]. The bioaccumulation of heavy metals in the different fish tissues has -been studied by several investigators (Filazi *et al.*, 2003; Ashraj, 2005; Zyadah, 2005; *catla* and Canpolat, 2006; Fernandes *et al.*, 2007; Uluturhan and Kucuksezgin, 2007; Fernandes *et al.*, 2008) [3, 14, 37, 7, 12, 13, 31].

In the present study, the LC50 values of CdNP of *Catla catla* are assessed to be 50ppm/Kg body weight. It is important mentioned here that toxicity of a CdNP is governed by many factors like water temperature, purity of the toxin and life stage of an organism etc., Hence, further studies were carried out with a dose of 20ppm CdNP/kg of body weight of fish.

3.2 Histological changes of eyes of major Indian carp *Catla catla* exposed to cadmium nanoparticles

CdNP induced structural changes in the eyes of the fresh water fish *Catla catla* stained by hematoxylin and eosin. In control, the retina is constituted by photoreceptors and pigment glial cells showed normal architecture. The photoreceptors project through the zona fasciculata to a group of three successively arranged optic ganglia: the lamina ganglionaris, the external medulla and the internal medulla. Two chiasms, the external and the internal, connect the lamina ganglionaris to the external medulla and the external medulla to the internal medulla respectively. On day 2, disorganization of corneal cuticle and medulla, on day 4 and day 5, further disorganization of corneal cuticle and formation of corneal cones and constriction of internal medulla and complete disorganization of basement membrane constriction of external medulla were evident (Fig.1).

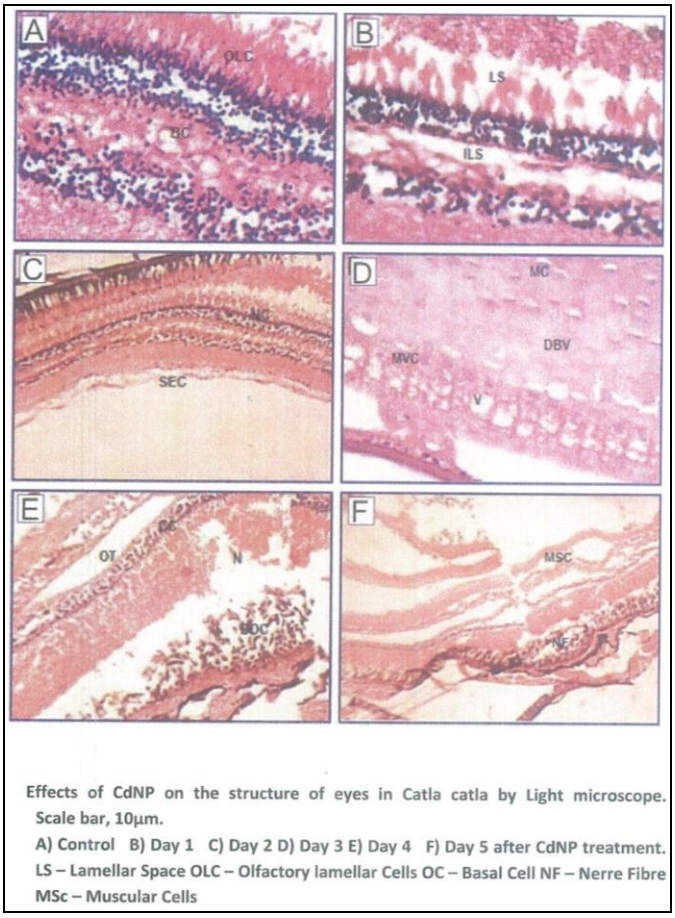

Fig 1: Effect of CdNP exposure on the structure of eyes of Indian carp, *Catla catla*

After exposure of CdNPs, Olfactory lamellae are formed due to folding of the epithelium and lamellae are arranged to form a cup shaped nucleus are observed at different levels of the epithelium. The supporting cells (SC) are broad and columnar in shape (10.25-12.55 µm) with prominent nucleus located in more superficial level in the epithelium and these cells located in close association to the olfactory rosette cells (ORCs). The basal cells (3.72-5.89 µm in diameter) are small, round with prominent central nucleus, situated at the basal part of the epithelium. Basal cells (BC) are of two types, globular basal cells and horizontal basal cells which are distributed throughout the base of the epithelium.

The olfactory bulb is round in shape and connected to the olfactory rosette by a very short olfactory nerve. Four distinct layers in the olfactory bulb are recognized from the periphery to center as nerve layer (NL), glomerular layer (GL), mitral cell layer (MCL) and granular cell layer (GCL) The olfactory organ of fish is lodged at the ethmoid region of the head and composed of many folds to form lamellae (Hara, 1975) [16]. The multilamellar olfactory organs of fish have an acute sense of smell in various aspects of life history and shows considerable diversity which reflects the degree of development and ecological habitats (Zeiske *et al.*, 1992) [36]. Modification of the olfactory system may occur through adaptation to a specific environment. Water entering the anterior inlet is conducted directly over the central part of the rosette from where it passes to the interlamellar spaces. This corresponds to the findings of Ojha and Kapoor (1973) [26] in the olfactory apparatus of *Labeo rohita*. The distribution of the sensory and non-sensory epithelia on the surface of the lamellae shows a great variety in different teleosts for adaptation to a specific environment (Yamamoto, 1982) [35]. In *Catla catla*, the sensory epithelium is restricted in the lingui form process whereas the broad lateral surface of the olfactory lamella is covered with nonsensory epithelium. This unique feature may be due to the fact that the sensory epithelium of lingui form process faces the flow of incoming water current and the receptor cells mobilizing different olfactory cues. Similar projections of the olfactory lamellae have been observed by Chakrabarti and Ghosh (2010) [8] in the olfactory epithelium of *Catla catla*. The ecological niche inhabited by a given species has an immense impact on celluar organization of the olfactory mucosa (Hara, 1994. Buck and Axel (1991) [6] reported that the olfactory processing commences at the apical tip of receptor cells. Hino *et al.* (2009) [18] postulated that fish could judge and detect the water soluble chemicals through the sensory receptor cells during water ventilation over the olfactory mucosa.

3.3. Histological changes of gills of major Indian carp *Catla catla* exposed to cadmium nanoparticles:

In the gill of control fish *C. catla* the primary gill lamellae are laterally compressed leaf like structures, attached alternately on either side of the interbranchial septum. Each primary gill lamellae consist of secondary gill lamellae on both the sides, which are perpendicular to its long axis. Primary gill lamellae comprised of a central core of cartilaginous rod and linings of epithelial cells are closely applied to gill ray. The damages, fusion and clumping of secondary gill lamellae were observed after 5 days of exposure. On exposure to CdNP for 5 days, erosion of epithelial cells, more mucus secretions and acute destruction to denticular structures were observed. In fish treated up to 5th days, the changes observed in the gill of *Catla catla* were swelling, fusion of lamellae, severe erosions of epithelial layer and high mucus secretion (Fig.2). The gill racker denticular structures were totally damaged and were uprooted from their bases. The primary gill lamellae (PGL) are laterally compressed flat leaf like structure on either side of the interbranchial septum. Each one of them bears a row of secondary gill lambellae (SGL) on both sides perpendicular to the long axis of primary gill lamellae. The secondary gill lamellae are highly vascularised and surrounded by a thin layer of epithelial cells. Between the two adjacent respiratory lamellae lie the interlamellar region.

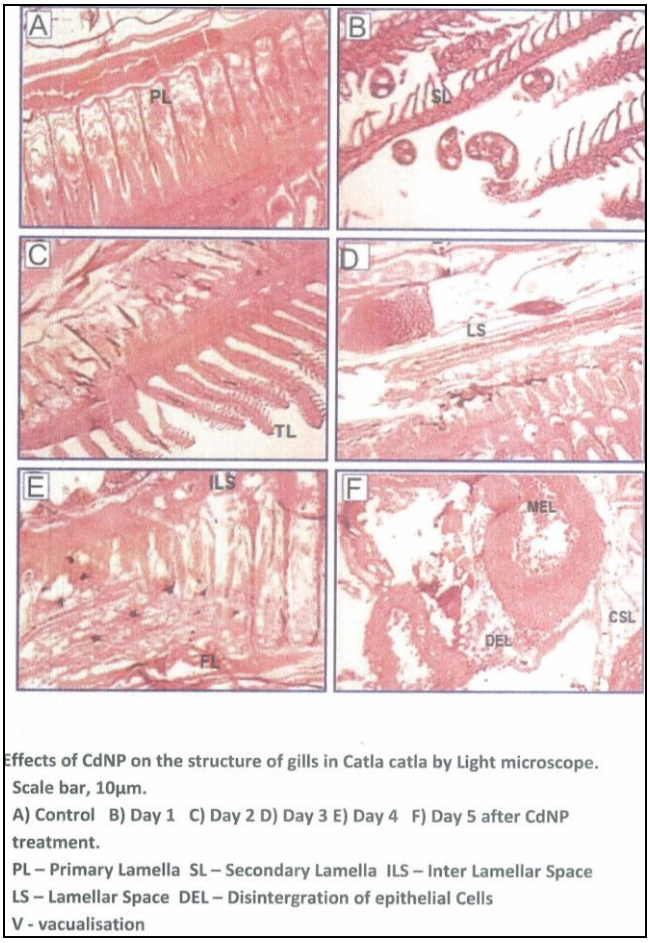

Fig 2: Effect of CdNP exposure on the structure of gills of Indian carp, *Catla catla*

In sublethal exposure of cadmium, the gill of *Catla catla* showed marked histological changes. Appreciable changes were noted in the histology of gill after 3rd day treatment including detachment of epithelium from the axis respiratory lamellae, fusion of secondary lamellae and vacuolization. The damage was more severe and progressive after 5th day of exposure. The gill epitheliums surrounding the axis of primary and secondary lamellae were damaged to a great extent clogging of respiratory lamellae, hypertrophy of gill filament and necrosis were observed. However, such changes were drastic to the extent that detachment of epithelium, hypertrophy and complete erosion of secondary gill lamellae were found in the treated fish.

Histopathological changes in the gills were observed in *Fundulus heteroclitus* exposed to cadmium (Gardner and Yevich, 1970) [15], in channel catlafish, *Ictalums punctalus*, experimentally and naturally infected with channel *catla catla* fish virus disease (Major *et al.*, 1975) [25], in *Thymallus arcticus* infected with ectoparasite monogenetic trematode, *Tetraonchus rauschi* (Wobeser *et al.*, 1976) [34], in blue gill, *Lepomis macrochirus*, exposed to monochloramine (Bass *et al.*, 1977) [4], in mummichogs, *Fundulus heteroclitus*. Fish gills are important target organs for heavy metals because of their large surface area in contact with the external environment and their thin membranes separating the internal medium from the external medium (Lucrecia Ferrari *et al.*, 2009) [24]. Thus, gills come immediately in contact with heavy metals dissolved in water provoking morphological and functional disturbances. Alterations in gill structure affect the normal functioning of vital physiological processes such as gas and ion exchanges, osmoregulation, excretion of nitrogenous wastes and acid-base equilibrium (Wendelaar Bonga *et al.*, 2008) [33]. Alvarado *et al.* (2006) [1] observed that exposure of turbot (*Scophthalmus maximus*) to Cu, Cd and Zn led to increase the total number of chloride cells in the gills.

3.4. Histological changes of muscles of major Indian carp *Catla catla* exposed to cadmium nanoparticles:

The photomicrograph of the muscle depicted the presence of normal myotomes with equally spaced muscle bundles. On exposure to sublethal concentration of CdNPs, marked thickening and separation of muscle bundles, haemolysis, necrosis, lesions with reduced compactness was observed and pronounced intramuscular oedema with minor dystrophic changes. Das and Mukherjee (2000) [10] in their studies on the fish *Labeo rohita* exposed to hexachlorocyclohexane, reported that due to the toxic effects of that particular substance at 1.73 ppm muscle tissue exhibited dystrophic changes with marked thickening and separation of muscle bundles in addition to severe intramuscular edema. Ramah (2011) [29] during his histopathological study on the effect of rice herbicides on Grass carp (*Ctenopharyngodan idella*), had observed the changes in muscle tissues are swelling and necrosis of muscle fibers.

After Exposure to CdNPs, muscle histopathological alteration was assessed semi-quantitatively by assortment the severity of alteration according to Bernet *et al.*, (1999) [5] into four significant reaction pattern (rp). In general, four major reaction patterns included circulatory disturbances, degenerative disturbances, proliferative changes and inflammatory changes. Each reaction pattern divided into several alterations. Circulatory disturbance included pathological condition of blood and tissue fluid that was considered in four major parts (hemorrhage, congestion). Degenerative disturbance included regressive alteration the lesion that can cause decreased muscle function or loss of muscle that was considered in four major parts (edema, degeneration of muscle fiber, atrophy of muscle fiber, necrosis, focal hyaline degeneration, increase perimysial space). Proliferative changes included alteration leads to an overactive cells or muscle function that was considered in seven major parts (hypertrophy of muscle cells, hyperplasia of muscle cells). Inflammatory changes that related to other reaction pattern were considered in one major part (lymphocyte infiltration of inter fasicular space) The fishes *Catla catla* collected from ponds shows histopathological alterations in their muscles due to alkaline environment, which are vacuolar degeneration in muscle bundles (VDM), edema between muscle bundles (EMB), splitting muscle fibers (SMF), development of necrotic areas (NA), degenerating muscle bundles (DM) (Fig.3).

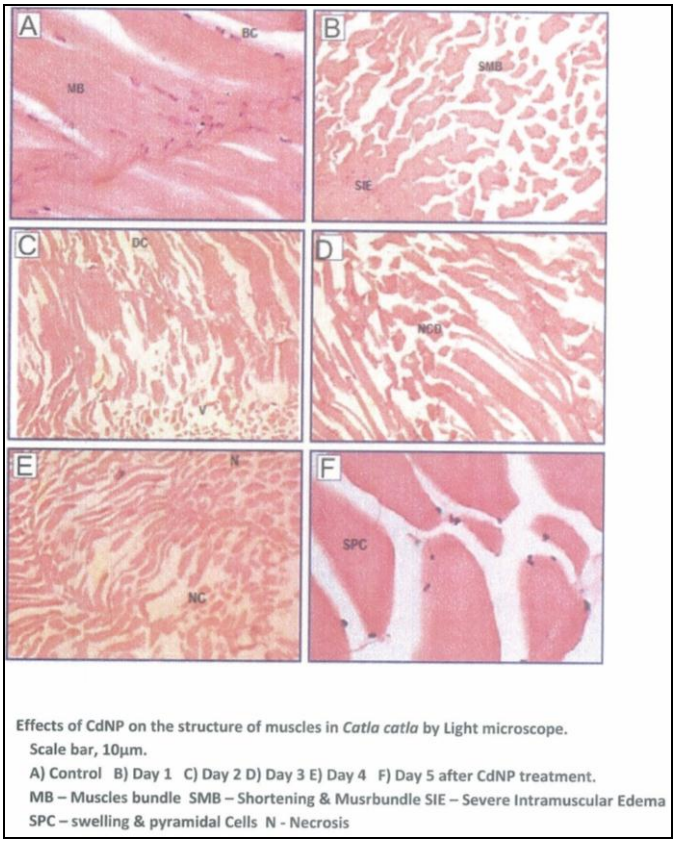

Fig 1: Effect of CdNP exposure on the structure of Muscles of Indian carp, *Catla catla*

The problem of heavy metals accumulation in aquatic organisms including fish needs continuous monitoring due to biomagnifying potential of toxic metals in human food chain (Das and Kaviraj, 2000; Laxi, 2005; Jayakumar and Paul, 2006; Kumar *et al.*, 2007; 2008; 2009) [9, 23, 19]. Due to the damage caused to the aquatic life, the pollution of freshwaters with a wide range of metals has become a matter of great concern over the last few decades.

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