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Histopathological alterations in gill, liver and muscle tissues of the freshwater fish, *Pseudetroplus maculatus* exposed to fullerene C_{60}

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Abstract
Among the engineered carbon nanomaterials, fullerene (C_{60}) is the most common 60-carbon atom molecule, which is arranged in a stable and symmetrical sphere with unique physico-chemical properties. Fullerene, a lipophilic compound possesses the ability to interact with all phospho-lipid membranes. The present study was aimed to evaluate that if fullerene C_{60} exposure could cause alteration in the histopathology of gill, liver and muscle tissues of the freshwater fish, *Pseudetroplus maculatus*. Fishes were exposed at 0.1mg/L of fullerene-C_{60} for 24, 48, 72 and 96 h maintaining the control group. At the end of every exposure period, fishes were sacrificed and tissues fixed in 10% buffered formalin. Histopathological analysis in control gill tissue showed normal gill architecture with compact lamellar gill epithelium, gill arches along with primary and secondary lamellae. Fullerene C_{60} exposed groups were shown with some structural alterations such as mucous deposition, aneurysm, loss of secondary lamellae, vacuolisation, curling of secondary lamellae and gill epithelial upliftment. Histoarchitecture of control liver showed normal hepatocytes with spherical nucleus and clear granular cytoplasm. However, exposure to fullerene C_{60} caused histopathological alterations such as vacuolisation, blood infiltration, necrosis and fusion of hepatic nucleus. Muscle tissue of control group showed normal muscle fibre with spindle nucleus. Exposure to fullerene caused disorganized muscle fibres with irregular or absence of nucleus. From the present findings it was clearly understood that fullerene C_{60} could cause adverse histopathological alterations in gill, liver and muscle tissues that could seriously affect the fish population near the exposure line

Keywords: Fullerene C_{60}, Histopathology, Gill, Liver, muscle, *Pseudetroplus maculatus*

1. Introduction
Nanomaterials are widely been used in industries as well as in scientific, research and medical areas. The applications of nanomaterials are rising immensely in various fields owing to the substantial benefits to the society. Concurrently, there is also an increasing concern over their potential toxic responses resulting from the increased use or accidental release of nanomaterials into the environment [1,2]. Fullerenes, a class of engineered carbon nanomaterials are widely used in commercial products and are expected to be one of the major contributors of environmental contamination in near future. Among carbon nanomaterials, fullerene C_{60} leads in the production globally and are unique as it possess caged configuration with the sixty carbon atoms arranged in the form of hollow sphere of cyclopentenes and cyclohexenes (Bucky ball) [3]. The identical shape of fullerene C_{60} favours its stability and persistence in water as colloidal aggregates that persist in nanometer size [4].
Fullerene C_{60} acquire dual properties as it has been shown to generate as well as quench the reactive oxygen species (ROS) in the biological systems [5, 6]. Differences in the size, shape, composition and characteristics of nanomaterials are considered as the factors responsible to influence toxicity in aquatic organisms [7]. Our previous studies have reported that fullerene C_{60} exposure altered the antioxidant defense system in the brain and hepatic tissues of the cichlid fish, *Pseudetroplus maculatus* [6, 8]. There are various reports stating the adverse effects of nanomaterials on the aquatic organisms [7, 9, 10]. The main reason behind the susceptibility of aquatic organisms, particularly fish, to the toxic effects of such environmental contaminants could be due to their frequent deliberate or unintentional release of toxicants into the aquatic ecosystem. Fish are considered as the most important components of aquatic ecosystems, and are also a primary exposure route to humans through the food chain.

Thus fish seems to be very sensitive to the exposure of wide variety of environmental contaminants that rise as accidental spillage from the production field, excessive usage, and effluents from industry as well as from various other sources. *Pseudetroplus maculatus* is an edible indigenous fish that occupies better ecological status within the food chain and are very sensitive to the surrounding environmental changes. *Pseudetroplus maculatus* is the most suitable model for laboratory studies as it is genetically stable having homogenous population and presence of adequate background data on the organism [11].

Histological investigation is a very sensitive tool to understand the structural modifications occurring in the tissues as a result of aquatic pollution. Gills are the site of respiration, osmoregulation and excretion, thus it is the first organ to which the pollutant comes to contact [12]. The organ most associated with detoxification and biotransformation is liver and therefore more vulnerable to toxicant-induced tissue damage [13]. Damages in muscle tissue are the direct indicator of aquatic pollutants in the body of organism. Therefore, it is necessary to study the histopathological alterations of various tissues of fish in detail. The present study was aimed to investigate the toxic effect of one of the nanomaterials, fullerene C_{60} by assessing the histopathological alterations in gill, liver and muscle tissues in the cichlid fish, *Pseudetroplus maculatus*.

2. Materials and methods

2.1 Animal

Healthy adult freshwater cichlid fish, *Pseudetroplus maculatus*, weighing 8.5 ±1.5 g and length 9 ± 1cm were collected from Kaloos Aquarium, Kottakkal, Malappuram district, Kerala, India. Fishes were transported to laboratory with least disturbance in well aerated polythene bags and acclimatized to the laboratory conditions for two weeks before experiment. During the period of acclimatization, fishes were fed with standard fish pellets and are maintained in well aerated large cement tank having a capacity of 40 L containing dechlorinated water. Animal was maintained in dechlorinated water with good lighting system (12: 12 h; light: dark) throughout the experiments and the health status of fish was also monitored. The physiochemical analysis of the tap water were analysed according to the method as described in APHA [14] with maintaining water temperature at 28 ± 2°C, dissolved oxygen at 8.5 and pH at 7.6

2.2 Chemicals

The carbon nanomaterial fullerene C_{60} (CAS No. 99685-96-8) of 98% purity was purchased from Sigma Aldrich, USA. Dimethyl sulfoxide (1%) was used as a vehicle control to disperse fullerene and which was sonicated in Sonics-Vibracell VX-400 at 35 Hz for 30 min at 3 sec pulse interval to attempt uniform dispersion before adding to the exposure tanks to reach 0.1 mg/ L. All other chemicals were of analytical grade and purchased from local commercial sources.

2.3 Experimental design

After acclimatization, healthy fish were selected, grouped with ten animals per group and treated with fullerene C_{60} at 0.1 mg/ L concentration (ie., 100µg/ L) for 24, 48, 72 and 96h maintaining control and vehicle groups.

2.4 Histopathology

At the end of treatment, gill, liver and muscle tissues from control and treatment groups were dissected, rinsed in physiological saline to remove blood and debris and fixed in 10% buffered formalin for 24h. Tissues were dehydrated in ascending grades of alcohol and were cleared in xylene till the tissues become translucent. Tissues were then transferred to molten paraffin wax for an hour for complete impregnating with wax. The tissue blocks were made then tissues were cut in sections of thickness 4 to 6 microns using rotary microtome. The sections were double stained with haematoxylin and eosin and mounted in DPX [15]. The slides were carefully examined and photographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope.

3. Results

Fullerene C_{60} exposed at 0.1 mg/ L concentration showed damage to tissues as gill, liver and muscle of the fish, *Pseudetroplus maculatus*. Control groups showed normal architecture of gill with compact gill epithelium, gill arches, primary and secondary lamellae. Fullerene C_{60} treatment for 24 h showed vacuolization, edema in primary lamella, fusion as well as loss of secondary lamellae**.** Exposure of nanomaterials for 48 h showed upliftment of gill epithelium, aneurysm in gill arches and primary lamellae, edema in primary lamellae, vacuolization in gill arch, shortening of primary lamellae and complete loss of secondary lamellae. At the end of 72 h of fullerene C_{60} exposure showed complete loss of secondary lamellae, upliftment of gill epithelium, aneurysm in gill arch and primary lamellae, vacuolization and hyperplasia in gill arches. Gill exposed to fullerene for 96 h showing upliftment in gill epithelium, aneurysm and degenerated primary lamellae (Figure 1a-f).

Histopathology of liver tissue in the control groups showed normal parenchymatous tissue with homogenous cytoplasm and spherical nucleus. After 24 and 48 h of fullerene exposure, cytoplasmic vacuolization was noted. The severity of vacuolization increased after 72 h of fullerene exposure and was followed with spindle shaped hepatic nucleus and erythrocyte infiltration after 96 h of toxicant exposure (Figure 2a-f). Muscle tissue showed normal architecture in both control tissues with compact muscle fibre and spindle nucleus. After fullerene C_{60} exposure was observed with split, thinned and shortened muscle bundles were the degeneration increased in time-dependent manner (Figure 3a-f)

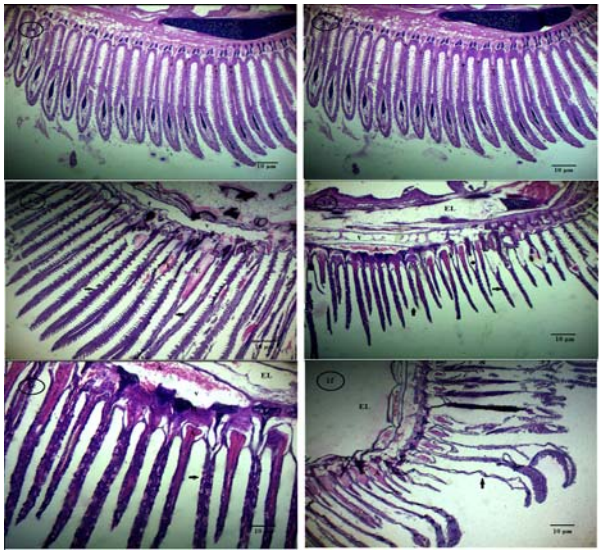

Fig 1a: Photomicrograph showing normal architecture of gill in control fish of *Pesudetroplus maculatus*. **b.** Normal histology of vehicle (DMSO-treated) group. **c.** Fullerene C_{60}-treated fish for 24 h showing vacuolization (V), edema in primary lamella (E), fusion of secondary lamellae (→), loss of secondary lamellae (←). **d.** Exposure for 48 h showing upliftment of gill epithelium (EL), aneurysm in gill arch and primary lamellae (A), edema in primary lamellae (*), vacuolization in gill arch (V), complete loss of secondary lamellae, shortening of primary lamellae. **e.** Complete loss of secondary lamellae (→), upliftment of gill epithelium (EL), aneurysm in gill arch and primary lamellae (A), vacuolization in gill arch (V) and hyperplasia in gill arch (H) exposed to fullerene for 72 h. **f.** Gill exposed to fullerene for 96 h showing upliftment in gill epithelium (EL), aneurysm in primary lamellae (A), degenerated primary lamellae (↑).

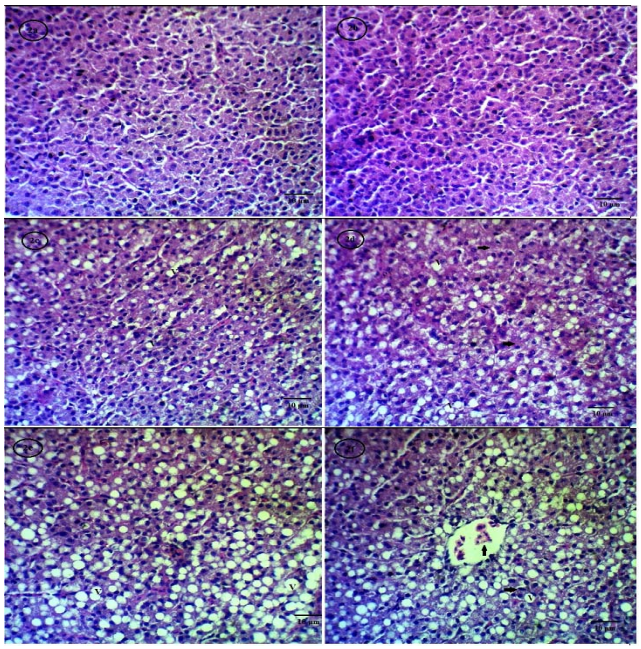

Fig 2a. Photomicrograph showing normal architecture of liver in the control fish, *Pesudetroplus maculatus*. **b.** Normal histoarchitecture in vehicle (DMSO-treated) group. **c.** Cytoplasmic vacuolization (V) in hepatic cells exposed to fullerene C_{60} for 24 h. **d.** Anucleated hepatocytes (→) and cytoplasmic vacuolization (V) after 48 h. **e.** Highly vacuolated hepatic cytoplasm after 72 h of fullerene exposure. **f.** cytoplasmic vacuolization (V), spindle shaped hepatic nucleus (→) and vacuolar enlargement with erythrocyte infiltration (↑) after 96 h.

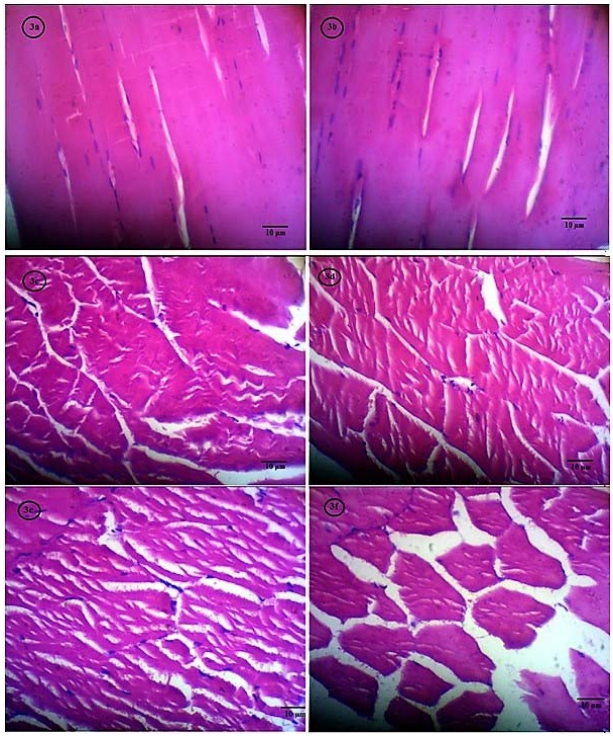

Fig 3a. Photomicrograph showing normal architecture of control muscle tissue with muscle fibre and spindle nucleus in the fish, *Pesudetroplus maculatus*. **b.** Normal histoarchitecture in vehicle (DMSO-treated) group. **c and d.** Degenerated muscle tissue with split muscle fibres after 24 and 48 h of fullerene exposure, respectively. **e.** Thinning of muscle fibres after 72 h. **f.** Thickened and shortened muscle bundles after 96 h of treatment.

4. Discussion

The physiological condition of the fish is one of the key factors that determine the health status of fish in aquatic ecosystem. Thus monitoring the physiological status of fish by using histopathological examination serves as an early warning signal to detect disease and long-term injury of cell or tissue due to aquatic pollution [16]. Histopathological alterations are closely related to other biomarkers of stress at cellular level since many pollutants undergo cellular metabolic changes in order to escape from toxicity. Exposure to zinc oxide nanomaterials has been shown to cause structural and functional changes in the fish, *Oreochromis mossambicus* [17]. In the present study one of the engineered carbon nanomaterials, fullerene C_{60} was exposed to the fish, *Pseudetroplus maculatus* at 0.1 mg/ L concentration for 96 h. Histopathological examination of tissues such as gill, liver and muscle were observed at 24 h interval for 96 h.

The fish gill is composed of four gill arches on each side of the buccal cavity. Each arch is composed of numerous gill filaments called primary lamellae with two rows of secondary lamellae that lie perpendicular to each filament [12]. Gills are liable to damage by any irritant materials which shows some alterations such as hyperplasia and hypertrophy of the epithelial cells, partial fusion of some secondary lamellae, excess mucous production as the primary defense mechanism on exposure to water borne toxicants [12]. Exposure to fullerene C_{60} for 24 h showed vacuolization, edema in primary lamella, fusion and loss of secondary lamellae. This could be the first line of defensive mechanism because edema with lifting of lamellar epithelium increases the distance across which waterborne pollutants diffuse to reach the

bloodstream [18]. After 48 h of fullerene treatment showed upliftment of gill epithelium, aneurysm in gill arch and primary lamellae, edema in primary lamellae, vacuolization in gill arch, complete loss of secondary lamellae and shortening of primary lamellae. Similar observations have been reported when the fish, *Pseudetroplus maculatus* was exposed to an environmental contaminant, chlorpyrifos [19]. Fullerene exposure for 72 h showed complete loss of secondary lamellae, upliftment of gill epithelium, aneurysm in gill arch and primary lamellae, vacuolization and hyperplasia in gill arch and this could cause a decrease in free gas exchange, thus affecting the general health status of the fish [20,21]. Gill exposed to fullerene for 96 h showed upliftment in gill epithelium, aneurysm and degeneration of primary lamellae, which are associated with the disturbance of blood flow in blood channels [22].

Liver is composed of parenchyma cells divided into irregularly shaped lobules separated by hepatopancreas and associated connective tissues. Hepatocytes are parenchymal tissue having homogenous cytoplasm with a large central or subcentral spherical nucleus. Liver is considered as the most important organ linked with detoxification and it is one of the organs most affected by contaminants in the water [23]. Several histological alterations were reported in liver of fishes when exposed to industrial pollutants [24]. Examination of histological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in field and experimental studies [25]. In the present study, fish exposed to fullerene C_{60} nanomaterials resulted in cytoplasmic vacuolization after 24 h. Cytoplasmic vacuoles of the hepatocytes has been shown to contain glycogen and lipids, which are related to the normal metabolic functions of the liver [13]. Thus vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of metabolic substances in the parenchymal cells and the rate of release into the systemic circulation [26]. In the present study the vacuolization found increased in time-dependent manner along with spindle shaped or anucleated hepatocytes. Hence, increased vacuolization of the hepatocytes are considered as a signal of degenerative process that suggests metabolic damage, possibly related to toxicant contaminated water [27]. Thus vacuole formation is a cellular defensive mechanism of hepatocytes against toxic substances that prevent from interfering with the biological activities of hepatocytes [28]. Exposure to copper nanoparticles has been reported with vacuolization in hepatocytes of *Cyprinus carpio* [29]. Diisononyl phthalate as well as fullerene C_{60} treatment has been shown to have spindle shaped nucleus in hepatocytes of *Oreochromis mossambicus* and *Oncorhynchus mykiss*, respectively [9, 30].

Fish muscle is an important valuable and recommended human nutrition possessing cardio protective effect due to the low content of fat and high content of proteins, minerals and optimal unsaturated fatty acids. Muscle of control groups showed normal architecture composed of elongated muscle fibers held together by connective tissues with spherical nucleus. In the present study fullerene C_{60} treatment showed progressive damage in the structure of muscle with increasing duration of exposure. The alterations noted include separation and thinning of muscle fibres after 48 and 72 h of fullerene exposure followed by shortening of muscle fibres at the end of 96 h treatment. Shortening and thickening of muscle fibres have been observed when the freshwater fish *Oreochromis mossambicus* was exposed to diisononyl phthalate [30]. Thus the degeneration of muscle fibres could be due to the depletion of glycogen or atrophy of muscle due to the toxic effects of fullerene C_{60} nanomaterials. The present observations showed that the severity of histopathological alterations in gill, liver and muscular tissue increased in time-dependent manner. Thus histopathology is sensitive parameter that provides complete assessment of health status of organisms when exposed to environmental contaminants.

5. Conclusion

Histological analysis confirmed that fullerene C_{60} nanomaterials uniformly caused alteration in the morphology of tissues such as gill, liver and muscle of the fish, *Pseudetroplus maculatus*. It can be concluded from the present study that exposure to single contaminant alone caused severe impact to the organism in laboratory condition. Thus it can be speculated that in natural ecosystem possessing combination of several pollutants could cause detrimental effects to the whole fish population and aquatic ecosystem.

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