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Behavioural and Histological variations in *Oreochromis mossambicus* after exposure to ZnO Nanoparticles

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Abstract
Nanoparticles are particles with one or more dimesnsions in the nanoscale range of 1-100nm. The ZnO NPs are characterized by XRD studies. Three different concentrations (30, 50 and 70ppm) of ZnO NPs are exposed to *Oreochromis mossambicus*. The general activity, social interaction and maintenance behaviour are severly disturbed in all treated groups especially in higher concentration (70ppm). Various tissues such as gill, muscle, brain, intestine and ovary are dissected out and histological analysis are performed. ZnO NPs treated gill tissues showed Necrotic lamella (NL), lamellar fusion (LFU), Abnormal Gill tip (AG), Proliferation of Chloride cells (PCC), excessive mucous formation (MU). Treated Muscle tissues showed Necrotic striated muscle (NSM), necrotic muscle fibres (NMF), shrinkage of muscle fibres. Vacoules (V) formation, Enalargement of Pyramidal cells (EPC) and necrosis are obsered in treated brain cells. Treated Intestinal tissues showed swollen goblet cells (SG), occurrence of Hyperplasia (H) and necrotic condition. Degenerated Late Vitellogenic oocytes (DLV) and formation of Vacuolation (V) and necrotic oocytes are observed in treated ovary tissues. Exposure of ZnO NPs disturbs the behavioural activity and degenerate various organs of the freshwater fish *O. mossambicus*.

Keywords: ZnO NP, XRD, Behaviour, histology, brain, ovary

1. Introduction
Nanoparticles are particles with one or more dimesnsions in the nanoscale range of 1-100nm (Farre *et al*., 2009) [15]. Zinc Oxide is widely used in a number of application like varistors (Rana *et al*., 2010) [32], UV lasers, gas sensors, photoprinting, electrochemical nanodevice, sunscreen lotion cosmetics and medicated creams (Ravichandrika *et al*., 2012) [33]. Due to its several properties such as goo transparency, high electron mobility, strong room temperature luminescence.

The entry of nanoparticles into an environment are from three major sources, such as anthropogenic sources, unintentional sources, production and usage of Engineered Nanoparticles (ENP) (Farré *et al*., 2009) [15]. The three main sources of ENPs released into aquatic ecosystems are summarized as follows: (a) Personal care products such as cosmetics and sunscreens, are made up of inorganic UV filters, including ZnO and TiO_2 NPs (Botta *et al*., 2011; Labille *et al*., 2010) [5, 23]. (b) Sewage: Availability of information is less about interaction of nanoparticles with water in sewage treatment process or how ENPs can be removed from waste water sludge (Brar *et al*., 2010; Kim *et al*., 2010) [6, 22]. (c) Anti-fouling paints: Nanoparticles used in anti-fouling paints for the prevention of attachment and growth of aquatic organisms on vessels hulls (Dineshram *et al*., 2009; Upadhyayula and Gaghamshetty, 2010) [12, 44,].

These engineered nanoparticles are inevitably released into the environment and have created significant concerns regarding their poteintial to cause adverse effects on the environment and human health (Handy *et al*., 2008; Savolainen *et al*., 2010) [19, 37]. Fish is the one of the abundant population of an aquatic environment. They are easily susceptible to any alteration in the physico-chemical characteristics of the habitat (Sadiq Bukhari *et al*., 2012) [36].

Fish gills, food and skin are generally recognized as three possible routes for any chemical substance from aquatic environment to enter the fish (Tao *et al*., 2000) [43]. NPs absorbed into the blood via gills and then reach the brain through the blood–brain barrier (BBB) via systematic distribution (Hu and Gao, 2010) [21].

Behavioral responses are useful indicators of pollution effects on aquatic animals (Eisler, 1979) [13]. Aggressive behavior is observed in reaction to exposure to of carbon nanotubes in rainbow trout (Smith *et al.*, 2007) [38]. Chafing has been widely observed among numerous families of teleost fish, including Cichlidae and Mozambique tilapia (Oppenheimer and Barlow, 1968; Barlow and Green 1970; Wyman and Walters-Wyman, 1985) [29, 4, 48]. It is considered as a maintenance behavior, with the primary goal to remove parasites or particles from the body surface of the fish (Galhardo *et al*., 2008; Wyman and Walters-Wyman 1985) [18, 48].

Histopathological studies are very sensitive and crucial parameter, which reflects the effect of toxicants on organ (Abdel-Warith *et al*., 2011) [1]. Histological biomarkers are closely related to others of stress since many pollutants either toxic or non toxic have to undergo metabolic activation in order to be able to culminate cellular change in the affected organism (Velkova-Jordanoska, 2002) [46]. As a tool for assessing endocrine disrupting effects in fish, histopathology has also been applied in other studies such as endocrine disruption in the ovaries and testes of zebrafish (Van der Ven *et al*., 2003) [45]. As a novel attempt, this study analyzed the behavioural and histological impact of ZnO NPs on the various tissues of freshwater fish *Oreochromis mossambicus*.

2. Materials and methods

2.1. X-ray diffraction (XRD) studies

Characterization of the nanoparticles is essential to provide the basis for understanding the properties of nanoparticles that determine their biological effects. The crystalline structure of ZnO NPs are analyzed by X'pert PRO PAN analytical X-ray diffractometer with Syn-Master 793 software, to confirm the component as well to determine the particle size. The lattice constants 'a' and 'c' of wurtzite structure can be calculated by using the relation (Suryanarayana and Grant Norton, 2005) [50].

$$\frac{1}{d^2} = \frac{4}{3}\left(\frac{h^2 + hk + k^2}{a^2}\right) + \frac{l^2}{c^2}$$

with the first order approximation (n=1) for the (100) plane, the lattice constant 'a' is obtained through the relation $a = \frac{\lambda}{\sqrt{3}sin\theta}$ and lattice constant 'c' is derived for the plane (002) by the relation $c = \frac{\lambda}{sin\theta}$. The unit cell volume (V) and bond length (L) (Wang *et al*., 2003) [51] are calculated by

$$V = \frac{\sqrt{3}a^2c}{2} = 0.866a^2c \qquad L = \sqrt{\left(\frac{a^2}{3} + \left(\frac{1}{2} - u\right)^2 c^2\right)}$$

where 'a' and 'c' are the lattice parameters and 'u' is positional parameter, by which each atom is displaced with respect to the next along the c-axis. The parameter 'u' can be calculated by the formula $u = \frac{a^2}{3c^2} + 0.25$.

The average crystal size of the samples are calculated after appropriate background correction from X-ray line broadening of the diffraction peaks of (101) plane using Debye Scherrer's formula (Chauhan *et al*., 2010) [52]

$$\text{Average crystal size } D = \frac{k\lambda}{\beta_{Dcos\theta}}$$

Where D-is the size in nanometers, λ is the wavelength of the radiation (1.5406Å for CuKα), k is a constant (0.94), β_D-is the peak width at half-maximum in radian along (101) plane and is Bragg's diffraction angle.

2.2. Experimental design

The freshwater fish *Oreochromis mossambicus* of both sexes (♂:♀) are collected from Cauvery River (lat. 10º 51′ and long. 70º 30′) in Tiruchirappalli district, Tamil Nadu (South India). Fishes are acclimatized in Environmental Research laboratory (Jamal Mohamed College, Tiruchirappalli) and the water parameters are maintained (APHA, 1998) [2]. Based on the LC50 results (Suganthi *et al.*, 2015a) [40]. Three different concentrations (30, 50, 70ppm) are selected for this study. After 96hrs of exposure, the control (without NPs) and treated groups are sacrificed.

2.3. Behavioural studies

Based on the toxicity, 30, 50 and 70ppm of ZnO NPs concentrations are selected for this study. Acclimatized fishes (n=10) are kept in the aquarium (glass tanks), covered with black non-transparent plastic to make 50% of the volume of the tank dark and 50% illuminated (Roques *et al.*, 2012) [34]. Behavioural sampling is carried out twice daily during the four days (Martin and Bateson, 1993). The daily total sampling effort was 60 min per aquaria (Galhardo *et al.*, 2008) [18]. The behaviour patterns are observed based on the various categories described by Baerends and Baerends-Van Roon, 1950, Diamond *et al.*, 1990, Sun *et al.*, 1994, Oliveira and Almada 1998a, Galhardo *et al.*, 2008 [3, 11, 42, 28, 18] (Table1).

Swimming activity (number of crossings from dark to light sections of an aquarium) is monitored under the hypothesis that a stressor alters light/dark preference (Maximino *et al.*, 2010) [27]. Control fishes are also maintained under same conditions without ZnO NPs. During observations, disturbances like loud noises, shaking of table, knocking or tapping of table, irregular light source, moving nearby or around the tank are avoided. The observers are sitting around the tank with minimal or less movements (Stalin *et al.*, 2013) [39].

Table 1: Brief description of the behaviour patterns and respective categories.

Categories of behaviour	Behavioural pattern	Description
General Activity[a]	Swimming	Fish progresses through the water with body undulation and fins movements.
	Hovering	Fish remains motionless over the substrate or the bottom of the tank.
	Inactive	Fish remains motionless in touch with the substrate or the bottom of the tank.
Social Interaction[a]	Non-specific interactions	Fish touches or swims very close to other fish, avoid light source.
Maintenance behaviour[b]	Chafing	Fish remove parasites or particles from the body surface during unfavourable environment
Others	Opercular movement[c]	Fish increases opercular rhythms and cough responses to compensate the loss of O_2 efficiency
	Comatose behavior[d]	Fish show no response to light, noise, and weak movement of caudal fin.

2.4. Histological studies

Gill, Muscle, Brain, Intestine and Ovary tissues are dissected and immediately fixed in Bouin's fixative for 48 h. The preserved tissues are processed by a routine histological method (Gurr, 1962), dehydrated in an alcohol series, cleared in xylene, infiltrated with liquid paraffin at 58º C, and finally embedded in paraffin blocks. The blocks are trimmed and sectioned at 5-8 mm thick cut on a rotary microtome (Weswox MT Chennai, India), are stained with the Harris' Hematoxylin and counter-stained with Eosin (H&E stain) mounted with DPX and observed under a light microscope (LEICA) (Suganthi *et al.*, 2015b) [41].

3. Results

3.1. XRD

The X-ray diffraction peaks of ZnO NPs are shown in Figure 1. The XRD peaks are located at angles (2θ) of 31.700, 34.348 and 36.183 corresponds to (100), (002) and (101) planes of the ZnO NPs, respectively. Similarly, other peaks found at angles (2θ) of 47.472, 56.534, 62.798, 66.312, 67.889, 69.109, 72.506 and 76.905 are corresponding to (102), (110), (103), (200) (112), (201), (004), and (202) planes of ZnO NPs. The standard diffraction peaks show the hexagonal wurtzite structure of ZnO NPs with the p63mc space group. This is also confirmed by the JCPDS data (Card no: 36-1451).

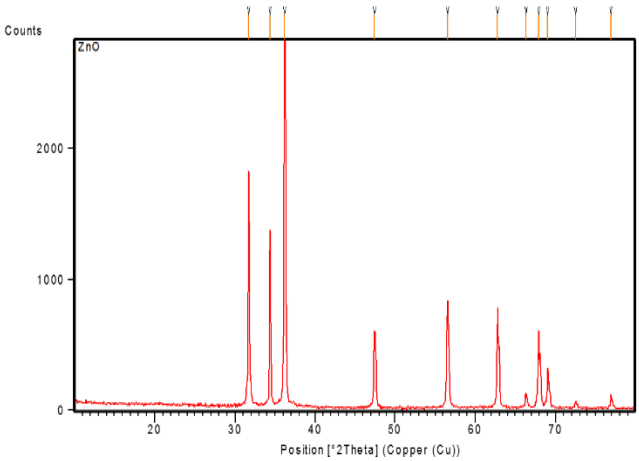


Fig 1: X-ray diffraction spectra of ZnO nanoparticles.

For ZnO NPs, values of the lattice parameters 'a' and 'c' are estimated 3.2566 Å and 5.2175Å respectively. There is a strong correlation between the c/a ratio and 'u'. The c/a ratio decreases with increasing 'u' in such a way that those four tetrahedral distances remain nearly constant through a distortion of tetrahedral angels due to the long-range polar interaction. The values of c/a and u are 1.9493 and 0.3798. The Zn-O bond length and average particle size of ZnO NPs is 1.9795Å and 47nm respectively.

3.2. Behavioural studies

In control fishes, black head, white cheek and throat and change their body colour in response to light source. Dark fins with orange-red free edges, the opercula remaining pale yellow. Dorsal and anal fin are rounded in females and pointed in males. Feeding activity occurs mostly during daytime. In all treated groups, the fishes are not respond to their light source and unware the entry of food particles into the tank. Treated fishes are hanging vertically in the water column, irregular jerky movements, continuous opercular movement and spits water. Dorsal and anal fin movements are reduced. The behaviour patterns are disturbed when the concentration of the ZnO nanoparticle increased (Table 2).

Table2: BehaviouralpatternsinControlandZnONPsexposed*O.mossambicus* (n=10)

Behavioural patterns	Control	ZnO NPs		
		30ppm	50ppm	70ppm
Swimming activity	-	+	++	+++
Hovering	-	++	++	+++
Inactive (motionless at the bottom)	-	+	+++	+++
Non-specific interaction	-	++	+++	+++
Chafing	-	++	+++	+++
Operuclar movement	-	++	+++	+++
Comatose behavior	-	+	++	+++
Fin movement (Initially)	-	++	+++	+++
Feeding inhibition	-	++	+++	+++
Mean	-	+	++	+++

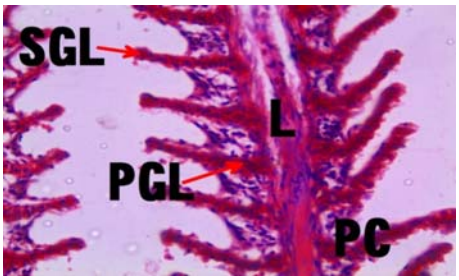
-: Normal (0%) activities; +: Mild (0-30%) disturbances; ++: Moderate (30-70%) disturbances; +++: Severe (70-100%) disturbances

3.3 Histological studies

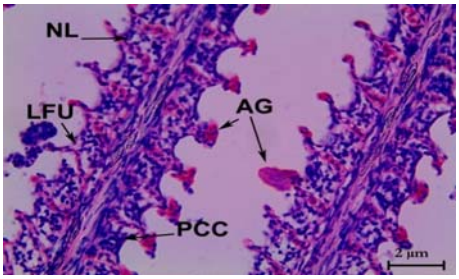
3.3.1 Gill

The control group gill tissues showed normal arrangement of gill rakes and lamella (L), no evidence of pathologies in primary gill lamella (PGL) and secondary gill lamella (SGL). In 30ppm, Necrotic lamella (NL), lamellar fusion (LFU), Abnormal Gill tip (AG), Proliferation of Chloride cells (PCC) are observed. In 50ppm, the occurrences of anomalies such as Necrotic lamella (NL), Lamellar fusion (LFU), Abnormal Gill tip (AG), Proliferation of Chloride cells (PCC) and also Mucous formation (MU) are observed. In 70ppm, occurrence of Necrotic lamella (NL), Lamellar fusion (LFU), Abnormal Gill tip (AG), Proliferation of Chloride cells (PCC), Mucous formation (MU) are increased when compared to control (Figure 2).

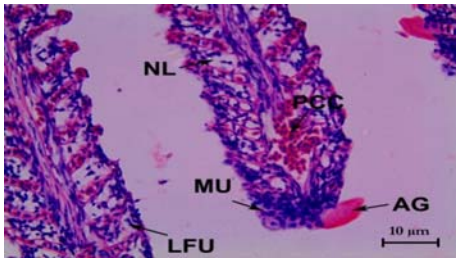
Control



30ppm



50ppm



70ppm

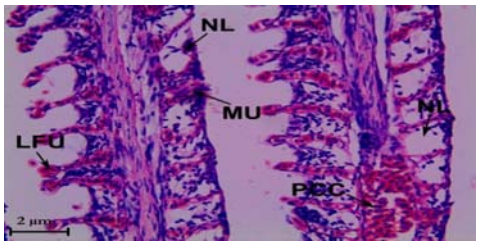
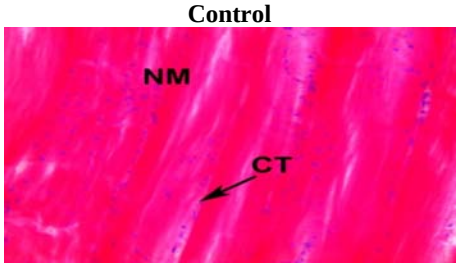


Fig 2: Gill section photograph of *O. mossambicus* in the control and 30, 50, 70 ppm ZnO exposed groups (×40).

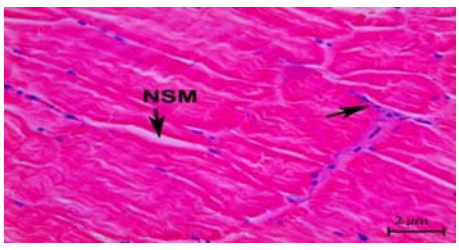
3.3.2. Muscle

Control muscle showed muscle fibers, fibril layers and connective tissue covers the muscle fibres, myofibrils and myofilaments. In 30ppm group, Necrotic striated muscle (NSM), necrotic muscle fibres (NMF), shrinkage of muscle fibres (SMF), pyknotic nuclei (small arrows). In 50ppm, NSM, NMF, SMF, pyknotic nuclei (small arrows) are observed. In 70ppm, tissue disintegration, NSM, NMF, SMF occurrences are increased (Figure 3) than other groups.

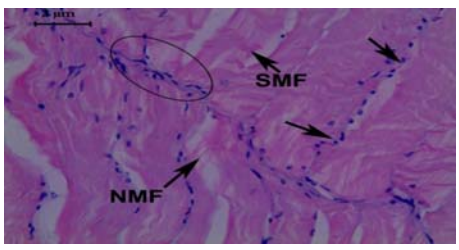
Control



30ppm



50ppm



70ppm

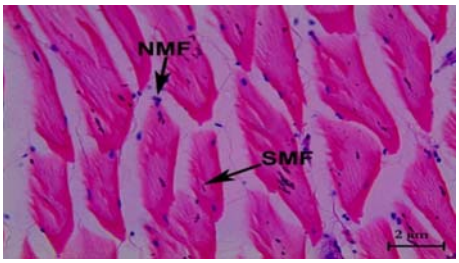
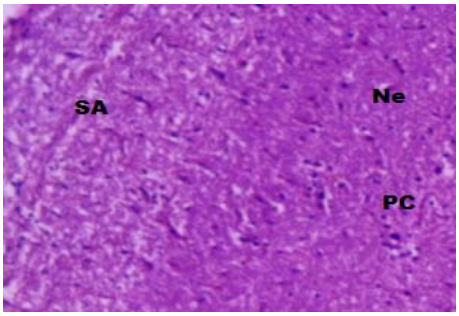


Fig 3: Muscle section photograph of *O. mossambicus* in the control and 30, 50, 70ppm ZnO exposed groups (×40)

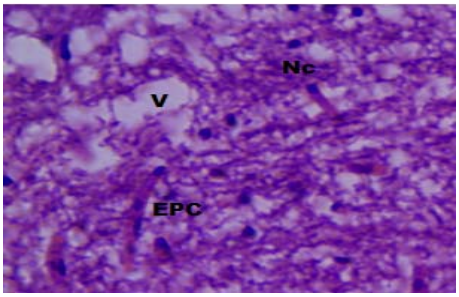
3.3.3. Brain

Control group fishes showed proper structural arrangement of cells in Septal Area (SA). Brain tissues showed the presence of normla neuronal cells (Ne), pyramidal cells and nissl substances. In 30ppm, structural arrangement are disturbed by formation of Vacoules (V), Enlargement of Pyramidal cells (EPC) and necrosis of brain cells. In 50ppm, structural deformities such as Neuronal cell degneration (NCD) and formation of vacoules in the septal area tissues, Dystrophic changes are also observed in brain tissues. In 70ppm, severe necrosis, vaoculation and binucleate formation in brain cells (Figure 4) are observed.

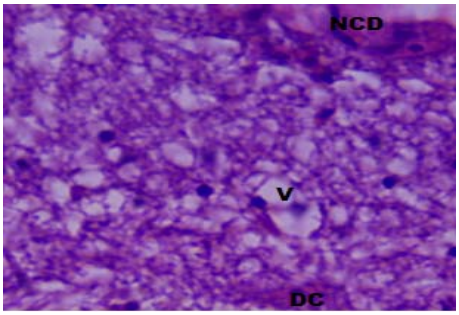
Control



30ppm



50ppm



70ppm

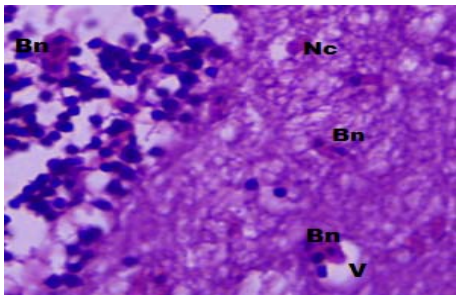
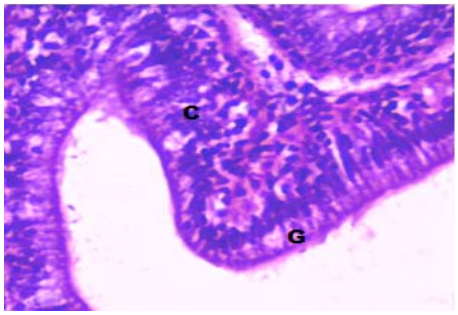


Fig 4: Histological sections of control and ZnO nanoparticles treated brain of freshwater fish *Oreochromis mossambicus*.

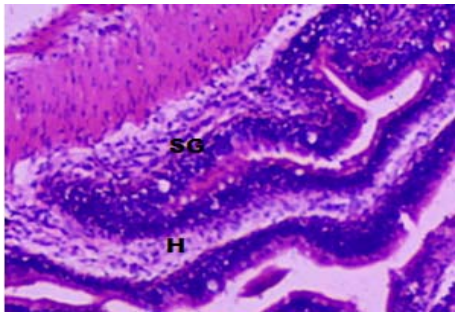
3.3.4. Intestine

Intestine of control group showed properly aranged Columnar (C) and Goblet (G) cells. In 30ppm, structural arrangement are disturbed by swelling of goblet cells (SG), occurrence of Hyperplasia (H) in intestinal villi. In 50ppm, structural deformities such as Vacoulation (V), swelling of goblet cells (SG) and Necrosis (N) are also observed. In 70ppm, severe necrosis, vaoculation in intestinal cells (Figure 5) are observed.

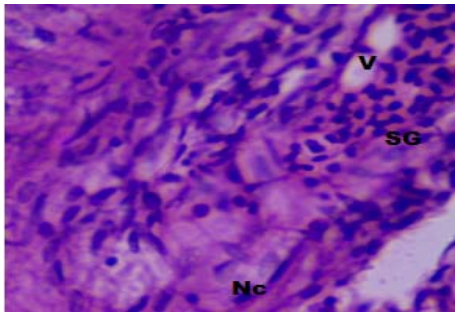
Control



30ppm



50ppm



70ppm

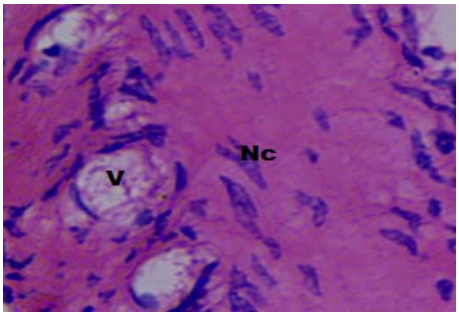
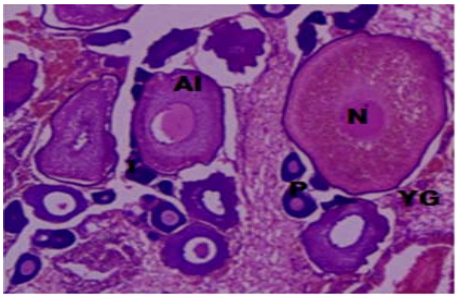


Fig 5: Histological sections of control and ZnO nanoparticles treated Intestine of freshwater fish *Oreochromis mossambicus.*

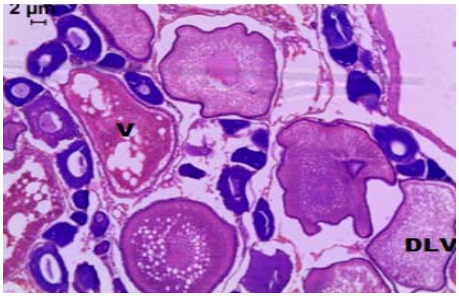
3.3.5 Ovary

Control group ovary tissues showed various stages of Oogenesis such as Primary oocyte (P), immature oocyte (I), Mature oocyte with prominent nuclues (N) and Yolk granules. In 30ppm exposed fishes showed Degenerated Late Vitellogenic oocytes (DLV) and formation of Vacoulation (V) in the cytoplasm. In 50ppm, necrosis of oocytes and also Vitellogenic fluid (Vf) in the ovarian parenchyma, possibly from degeneration of mature vitellogenic ooctyes are observed. In 70ppm, Degenerated Late Vitellogenic oocytes (DLV) and formation of Vacoulation (V) in the cytoplasm are observed (Figure 6).

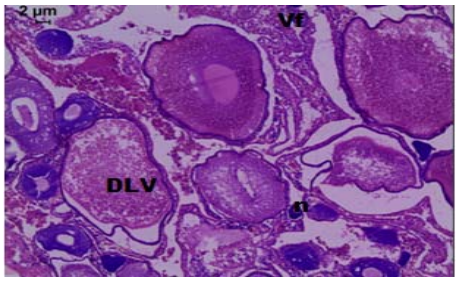
Control



30ppm



50ppm



70pppm

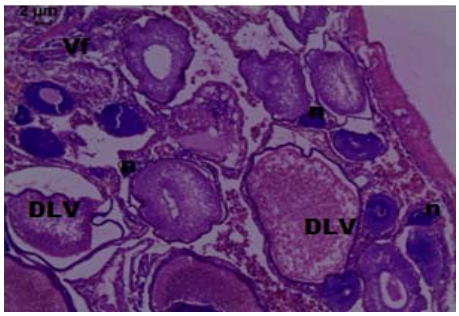


Fig 6: Histological sections of control and ZnO nanoparticles treated ovaries of freshwater fish *Oreochromis mossambicus.*

4. Discussion

The abnormal behaviors (e.g., hanging vertically in the water column for a few seconds–minutes, swimming in small circles near the water surface) are qualitatively described in fish exposed to TiO_2 NPs (Federici *et al.*, 2007; Hao *et al.*, 2009) [16, 17, 20]. Chervova (1997) [8] concluded that caudal fins are among the most sensitive zones for damage, due to aggressive behavior, in White Sea cod (*Gadus morhua marisalbi*) and steelhead salmon (*Salmo mykiss*). The dorsal and caudal fins are fragile and thin progressively tears. This resulted in short spurts of swimming (Farlinger and Beamish, 1977) [14]. The whole body involving in twisting movements (Christine and Gokhale, 2000) [9].

Ecologically speaking, aberrant locomotion behavior and activity level could cause negative impact on predator–prey interactions, reproductive behavior, migration, and dispersal and thus decrease fitness of fish (Little and Finger, 1990; Vieira *et al.*, 2009) [24, 47]. The behavioral endpoints are more sensitive than the others such as hatchability and survival (Chen *et al.* 2011) [7] to detect the toxicity of NPs in fishes.

Histopathological changes of gills are observed in TiO_2 NPs exposed rainbow trout (Federici *et al.*, 2007) [16, 17]. Rajakumar and Rahuman (2012) [31] are observed the several histopathological conditions in 50 mg/L Ag NPs treated fish gill such as mild congested blood vessels, fused primary lamellae and hyperplasic branchial arch. In 25 mg/L Ag NPs concentration, the muscle tissue exhibited dystrophic changes with marked thickening and separation of muscle bundles in addition to severe intramuscular oedema (Rajakumar and Rahuman 2012) [31].

Mild vacuolar changes with empty spaces appeared due to increased concentration and duration of zinc toxicity to *Labeo rohita* (Loganathan *et al*., 2006) [25]. Das and Mukherjee (2000) [10] reported that hexachlorocyclohexane is neurotoxic and induced vacuolation of brain parenchyma and moderate swelling of pyramidal cells of the cerebrum and opined that vacuolation may have been due to glycolysis leading to microsomal and mitochondrial dysfunctions. Vacuolization in brain tissue may be the result of glycolysis leading to microsomal and mitochondrial dysfunctions. Severe necrosis of neuronal cells in the cerebrum indicating loss of nissl substances (Patnaik *et al*., 2011) [30].

Younis *et al*. (2013) [49] reported the intestinal tissue in the $CdCl_2$ treated groups is characterized by increased degenerated nuclei and apoptosis in crypts of Lieberkuhn. Goblet cells increased in all treated groups, indicating a defense mechanism against the severe pathological changes. Spontaneous lesions of oocytes can also occur randomly under normal conditions, as reported in zebrafish studies, and this is a phenomenon of fish pathology that has been broadly investigated (Rossteuscher *et al*., 2008) [35]. Under the control conditions, the females did not show a susceptibility to spontaneous oocyte atresia, as shown by the low frequency of atresia, compared to exposed organisms. Degeneration of mature vitellogenic oocytes due to chemical exposure (Van der Ven *et al*., 2003) [45].

5. Conclusion

ZnO nanoparticles exposure caused severe impact on behaviour and tissues of *Oreochromis mossambicus.* As a novel study, the observed behavioural changes create life threatening conditions to the fishes. Histological abnormalities observed in gills, muscle, brain, intestine and ovaries tissues of exposed group, thus their physiological, secretory and absorption, endocrine and reproductive activities are disturbed. Increased concentration of ZnO eventually increases the abnormalities in *Oreochromis mossambicus*.

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